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ブリ (*Seriola quinqueradiata*) の産卵, 回遊生態及び その研究課題・手法について

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On the Spawning and Migration of Yellowtail *Seriola quinqueradiata* and Research Required to Allow Catch Forecasting

Toshihiro YAMAMOTO, Shingo INO, Masahiro KUNO, Hideo SAKAJI,
Yoshiaki HIYAMA, Tatsu KISHIDA and Yukimasa ISHIDA

Abstract Since the yellowtail *Seriola quinqueradiata* is an important fish stock for the fixed net and purse seine fisheries around Japan, accurate forecasts of the landing at each location and fisheries management are required. In this paper we review the spawning and migration biology of the yellowtail and discuss what research is required to achieve these objectives. Spawning biology of yellowtail was reviewed from the growth of larvae and the maturation of spawning adults as well as from past data on egg and larval net surveys. The migration pattern differed with the growth stage and a large scale migration from north to south occurs after maturation. The area of distribution especially in the wintering season seems to depend on the marine environment. We consider that clarification of the relationship between the migration and environmental conditions is necessary to improve the accuracy of forecasts of landings and we discuss the research required to enable effective forecasts.

Key words: *Seriola quinqueradiata*, yellowtail, spawning, migration, environment

第1章 はじめに

ブリは, 神事にも使われるなど, 古来より我が国の人々に親しまれてきた魚である。漁業養殖業統計年報(農林水産省統計部)によれば, 平成14年のブリ養殖魚生産は10万8千トン, ブリ類海面漁業生産は5万1千トンと, ブリ鮮魚生産に養殖魚の占める割合が高い

が, 冬季に漁獲される大型のブリを始め, 各地の特徴的な漁獲物によって, 海面漁業漁獲物(天然魚)は地域文化・産業の重要な位置にある。

ブリは, 養殖種苗用のモジャコ採捕から大型ブリまで, 東シナ海から北海道沿岸域の広域で定置網, まき網, 釣りなど多様な漁業によって漁獲される。漁獲統計上, ブリはカンパチ, ヒラマサと合わせてぶり

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類として計上されることが多いが、ブリが大きな割合を占めるので、ぶり類の漁獲量変動傾向をブリのそれと見ることができる。全国のぶり類漁獲量は、統計値が得られる1989～2003年の間に、増減を繰り返しながら、全体的には増加傾向を示している（阪地，山本，2006）。しかし，日本海では1970年代から90年代半ばまで減少傾向が引き続くなど，減少傾向が著しい年代もあって（村山，北原，1992），単調に増加傾向が続いてきた訳ではない。また，海域による変動傾向の差異も認められる（木幡，1987；檜山，1990；原，村山，1992）。

このような漁獲の変遷の中で，かならずしも有効な漁獲利用が行われていないのではないかと，あるいは，漁獲圧が資源変動に影響を与えているのではないかという問題意識が示されている（例えば，加藤，渡辺，1985）。とりわけ，モジャコ採捕は漁獲尾数が大量であるため，資源に与える影響が大きいのではないかと懸念があり，「モジャコ採捕のブリ資源に及ぼす影響に関する研究（農林水産技術会議事務局特別研究費）」が行われた（農林水産技術会議事務局，1967）。このプロジェクト研究は，モジャコの分布・移動やブリの年齢・成長などの生物学的知見を明らかにしたが，モジャコの漁獲圧について明確な結論は得られなかった。現在モジャコ採捕数は最盛期よりも減少したものの，毎年多量に採捕されており，その資源に与える影響の評価を望む声も強い。また，ブリは，まき網漁業と定置網漁業といった漁業間調整の中で，主要な漁獲対象魚種として議論されることも多い。資源評価によって漁獲圧が適正かどうかを判断し，有効な漁獲方策を提案することが望まれる。

一方，ブリの漁況が海況に大きく影響されることが，古くから意識されてきた（例えば，山寺，1935）。ブリを対象とする漁業は，能動的にブリの群れを追いかけるより，地域的な来遊状況に漁模様が規定されてしまう場合が多いので，精度の高い漁況予測が求められてきた。また，漁獲量や漁獲物年齢組成などの漁業情報から資源の増減傾向を知るためには，漁況に及ぼす海況の影響をよく理解して検討することが必要である。

原（1990a）と北原，原（1990）は，日本海に來遊するブリの量の経年変動を表す指標値として，來遊量指数を提案した。その導出過程で，年*i*の漁場*j*におけるブリ漁獲量 $C_{i,j}$ を以下のように表した。ただし， f は漁獲率， $E_{i,j}$ は*i*年の漁場*j*-1から*j*への逸散率， P は來遊量。

$$C_{i,j} = f_{i,j} \left(\prod_{k=1}^{j-1} E_{i,k} \right) P_i$$

両辺に対数をとって，

$$\log C_{i,j} = \log f_{i,j} + \sum_{k=1}^{j-1} \log E_{i,k} + \log P_i$$

を得る。この式は，漁獲量の変動が，ブリの來遊量の変動と，漁獲率や逸散率に関わる量の変動とに分離できることを示す。漁獲率や逸散率は海洋環境に大きく左右されると考えられる。また，日本海といったある海域への來遊量は，全体の資源量や回遊生態に依存するとともに，海洋環境の影響を大きく受けることが想定される。さらにこの式は，來遊量に関する情報と，漁獲率や逸散率に関わる要因としての海洋環境の情報から漁況を予測することの骨格を示している。

ブリの漁場への來遊状況が海洋環境にどのような影響を受けるかを検討した研究は，数多く存在する。三谷（1965a, b, c, 1968）は，航空観察による流れ藻量からモジャコ資源量を推定することを試み，その中で，流れ藻の出現状況が年によって大きく異なることを観察し，海流や潮目への集積が影響すると考えた。原（1990b）は，漁獲量資料と水温データから，日本海において春と夏に水深100m層の水温が10℃以上の海域が陸岸に沿って北へ広がる年ほど，より多くのブリ1歳魚が能登半島以北へ來遊すると推測した。さらに原（1990c）は，漁獲資料と対馬海峡西水道の水位差データを解析し，対馬暖流の流量が増加すれば，それに乗って日本海を北上するブリの量が増大する可能性は高いと考えた。三谷（1960）は，約24～27年の周期で，日本海北部と東シナ海の漁獲割合が増減することを示し，その原因は対馬暖流勢力の強弱と密接な関連があるとした。

このように，來遊量及び漁獲率や逸散率に関わる量の変動は，成長段階としては稚魚から成熟魚まで，時間的には数日から数十年，空間的には我が国周辺沿岸海域の大部分に渡る変動が入り混ざっているのである。これら，ブリの回遊生態とその年変動様式，それに影響を与える海洋環境の，資源評価や漁況予測において現在問題となる重要な点について，解決を図る研究を進めることが望まれる。

ブリの回遊と海洋環境の関係について新たな研究の展開を図るためには，数多く存在する既往知見を整理して問題点を抽出し，その解決方法を探ることが必要である。そこで本報告では，第2章で「産卵場，初期生活史に関する既往知見」を，第3章で「移動回遊に関する既往知見」を整理する。第4章では，これら既往知見の整理から導き出される，回遊と海洋環境の関係についての研究を進めていくうえで必要となる課題を論じ，第5章では，それらの研究を進めるための手法について，特に各種最新手法の適用可能性の観点から検討する。

本稿を作成するにあたり第1章は檜山義明、第2章は山本敏博、第3章は井野慎吾、久野正博、第4章は岸田 達、石田行正、第5章は阪地英男、山本敏博、並びに井野慎吾が主に担当した。本稿をまとめるに当たり有益な助言、指摘を頂いた東北大学大学院南 卓志教授に深謝する。

第2章 産卵場、初期生活史に関する既往知見

2-1 産卵海域と産卵期

ブリの産卵生態に関する知見は、1952年に始まった「対馬暖流水域の開発に関する調査（対馬暖流開発調査）」（農林水産業技術振興費による農林漁業試験研究補助事業）以前は非常に乏しく、稚魚の採集海域や各沿岸海域で採集された親魚の生殖腺の性状が報告され（代表的なものは木村（1952））、これらの情報から産卵海域と産卵期に関する推察が行われていたに過ぎない。一方、「対馬暖流開発調査」までの知見については三谷（1960）がその論文の中でまとめているが、本項では必要と判断される知見について再度引用し、また、現在までの知見を「東シナ海（薩南海域含む）～日本海」「東シナ海～太平洋」に分けて概観する。さらに、「成熟期にある親魚の地理的分布と出現時期」、「卵仔稚魚の地理的分布と出現時期」、「産卵好適水温」の項目について整理し、「産卵海域と産卵期の推定」を行って問題点を整理した。

東シナ海～日本海 1952年に「対馬暖流開発調査」が開始されて以来、ブリの卵や仔稚魚が多数採集され、また、大型ブリの成熟状況が報告され、東シナ海および日本海における産卵期並びに産卵海域に関する具体的資料が提供された。今井（1954, 1955）は薩南海域におけるブリ稚魚の出現傾向を明らかにし、加藤（1954, 1955）は京都府沖合におけるブリ稚魚の出現を明らかにした。内田（1954）、内田ら（1958a, b）は長崎県男女群島女島、並びに五島列島南端玉之浦沿岸水域で天然卵の採集を報告した。川村（1955）は1950年6月～1954年12月の5ヵ年間にわたる対馬海峡での稚魚採集結果を整理して、同海域におけるブリ仔稚魚の出現傾向を明らかにした（Table 1）。内田（1955）は1952～1955年にわたる表層曳稚魚網による卵仔稚魚の採集資料をまとめ、ブリの産卵は対馬暖流域では九州西岸に限られるようであるが、仔稚魚の出現は九州西岸では2～7月（盛期は4～6月）、山陰沖では4～6月、北陸では6～8月となっていること、また、九州西岸での仔稚魚の出現期間が長く3、4月に北緯30度以南の海域でも採集されていることから、産卵場がかなり南方にも広がっていることを推測した。

Shimomura and Fukataki（1957）および深滝（1958）は、1953年1月～1956年7月の間に対馬暖流開発調査の稚魚網で採集された資料に基づいて、日本海側におけるブリの産卵期や産卵場を明らかにし、それまでの調査結果を総括した（Table 1）。「対馬暖流開発調査」以降、内田、庄島（1958）は1957年3月から1958年3月の間に北部九州の沿岸（津屋崎）で流れ藻採集を行い、同海域では成長したモジャコ（45-108mmTL）が5～6月に採集されることを明らかにした。千田（1962a, b）は1957年5月から1961年11月の間に表層稚魚網を用いた隠岐周辺海域におけるブリ仔稚魚の出現傾向を示し、同海域では5～8月に出現し6～7月が中心であること、仔稚魚は沖合域にはみられず沿岸域でのみみられることを明らかにした。沖山（1965）は、1962年6月から1963年5月に佐渡海峡で採集を行い、7月にわずか1個体であるがブリ稚魚を採集している。池原（1977）は、1975年2～8月と1976年4～8月に佐渡海峡で表層稚魚網を用いて採集を行い、1975年は7、8月に4個体、1976年も7、8月に12個体を得ている。1978年からは日本栽培漁業協会が国の委託を受けて「天然ブリ仔資源保護培養のための基礎調査実験」を開始し、東シナ海から西日本の日本海にかけての産卵海域及び産卵期に関する資料が補足された。山陰西部の山口県沿岸域ではブリ仔稚魚が5月上中旬から出現し始めることが藤田、森（1982）によって明らかにされた。一方1980年代以降、東シナ海で大中型まき網漁業によるブリ親魚の漁獲が急激に増加したが、村山（1992）は、1988年3、4月、及び1989年3～5月のこれら漁獲統計資料と漁獲されたブリ親魚の生殖腺および年齢を査定することによって、ブリの産卵場が大陸棚縁辺に形成されること、水温の上昇とともにその海域が大陸棚に沿って北上することを示し、また産卵に参加する親魚の年齢が3歳以上で構成されていることを明らかにした。また、村山（1992）は東シナ海に形成される産卵海域のGEK（電磁流速計）資料を解析することによって、黒潮流域およびその縁辺部で産卵された卵仔稚魚は比較的安定して太平洋側へ補給されるが、対馬暖流域に産卵された卵仔稚魚は産卵時期によって太平洋へ輸送されたり、日本海へ輸送されたり、大きく変化することを推測した。さらに、村山（1992）は山陰沿岸、九州西岸～紀伊半島にかけて加入したモジャコの日輪分析（飼育による成長と飼育日数との関係を数式化して、全長からふ化日を推定）からそのふ化日を明らかにし、GEKの解析結果と照合して、2、3月に東シナ海で生まれた卵仔稚魚は太平洋側に、一方、4、5月に産卵された仔稚魚は対馬暖流側に輸送される可能性が高いことを示唆

Table 1. Monthly change of frequency occurrence of eggs and larvae by area. The notations used for areas are the same as Fig. 1. Upper and lower lines of each area indicates larvae and eggs, respectively. Occurrence frequency; Δ : 1-several, $\bigcirc$: dominant, $\odot$: predominant. Literatures by Uchida *et al.* (1958a, b) and Matsuda (1969) referred to eggs as well as larvae.

Area	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Sources
A							$\odot$	Δ		Shimomura and Fukataki (1957), Fukataki (1958), Okiyama (1965), Ikehara (1977)
B					$\odot$	$\odot$	$\bigcirc$			Katoh (1954, 1955), Shimomura and Fukataki (1957), Fukataki (1958)
C			Δ	Δ	$\odot$	$\odot$	Δ			Shimomura and Fukataki (1957), Fukataki (1958), Senta (1962a, 1962b)
D		Δ	$\odot$	$\odot$	$\odot$	Δ	Δ	Δ		Kawamura (1955), Shimomura and Fukataki (1957), Uchida <i>et al.</i> (1958a, 1958b), Fukataki (1958), Uchida and Shojima (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Sakakura and Tsukamoto (1997), Cho <i>et al.</i> (2001), Yamamoto <i>et al.</i> ^(*1,*2)
E		Δ	Δ	Δ	$\bigcirc$	Δ				Shimomura and Fukataki (1957), Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Seikaiku National Fisheries Institute (1966, 1967, 1969), Yamamoto <i>et al.</i> ^(*1,*2)
F		Δ	Δ	Δ						Shimomura and Fukataki (1957), Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Seikaiku National Fisheries Institute (1966, 1967, 1969), Yamamoto <i>et al.</i> ^(*1,*2)
G		Δ	Δ							Shimomura and Fukataki (1957), Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Seikaiku National Fisheries Institute (1966, 1967, 1969), Yamamoto <i>et al.</i> ^(*1,*2)
H		Δ	Δ	$\odot$	$\odot$	Δ	Δ			Uchida (1954), Shimomura and Fukataki (1958), Fukataki (1958), Matsuda (1969), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Hanaoka (1995), Ishida <i>et al.</i> (1997), Seikaiku National Fisheries Institute (1966, 1967, 1969), Yamamoto <i>et al.</i> ^(*1,*2)
I		Δ	$\odot$	$\odot$	Δ	Δ				Shimomura and Fukataki (1958), Fukataki (1958), Matsuda (1969), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Hanaoka (1995), Ishida <i>et al.</i> (1997)
J		Δ	$\odot$	$\odot$	Δ	Δ				Hattori (1964), Matsuda (1969), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Ishida <i>et al.</i> (1997)
K			$\odot$	$\bigcirc$	Δ		Δ			Hattori (1964)
L			Δ	$\bigcirc$	Δ					Hattori (1964)
M			Δ	$\bigcirc$	Δ	Δ				Odate (1962, 1967), Safran (1990), Safran and Omori (1990)
N			Δ	Δ	$\bigcirc$	Δ				Odate (1962, 1967), Safran (1990), Safran and Omori (1990)

した。上原ら (1998) は、村山 (1992) が行った漁獲統計資料を用いた方法で、1994～1996年の東シナ海におけるブリの産卵場も東シナ海の大陸棚内縁に沿って形成されていることを確認した。Cho *et al.* (2001) は記録の少ない朝鮮半島東南岸におけるブリ仔稚魚の出現傾向を調べ、直径0.8mのタモ網で採集される小型の仔稚魚は5月に限って採集され、また、口径1.5m×1.5mの表層トロール網で採集されるやや成長した稚魚は5月を主として7月まで採集され、さらに、袖間30m×網丈10mの小型巻き網で流れ藻とともに採集される稚魚から幼魚(全長135mm以下)は5～7月、主として5、6月に採集されることを明らかにした。山本ら^{*1, *2}は、2001～2003年の2～4月に薩南海域から東シナ海全域にかけてボンゴネット斜め曳き採集、ニューストンネット採集を行い、ブリの産卵海域が東シナ海の大陸棚内縁に沿って見られること、仔稚魚は水温20℃等温線に沿って高密度でみられることを明らかにした。また、ブリ仔稚魚が表層性であることを薩南海域～太平洋沿岸では服部 (1964) が報告したが、ボンゴネットとニューストンネットの採集結果を比較して改めて明らかにした。

東シナ海～太平洋 服部 (1964) は、1954年4月から1959年12月の間に薩南海域からベーリング海にかけて採集された卵仔稚魚の情報についてとりまとめ、太平洋岸でのブリ稚魚の出現は4～6月および9月で、薩南から房総半島までの主として沿岸部でみられ、全長10mm以下の個体が4、5月を中心に出現して6月には減少することから主産卵場は黒潮上流海域・本邦南海域にあると推定した。また、北緯35度以北の東北・北海道の沿岸では分布がみられないことも明らかにした。さらに、水平曳き採集と鉛直曳き採集結果を比較して仔稚魚が極表層でのみ採集され、鉛直曳きでは、卵仔稚魚がほとんどされないことから、ブリの産卵は表層で行われ、仔稚魚も表層性であることを明らかにした。また、松田 (1969) は、1954年1月から1963年12月までの日向灘を中心とする海域と1966年4月から1968年3月までに薩南海域から紀南沿海域で行った表層稚魚網採集による仔稚魚の出現傾向を調査し、ブリの卵は1～5月、仔稚魚は3～7月に出現し、年間を通じ4～5月に90%以上が採集されることを明らかにしている。小達 (1962) は、1949～1960年の間に東北海区(道南～房総沖)で表層稚魚網によるブリ仔稚魚の採集結果から、同海域では4～7月に採集され、6月に最も多く採集されることを明らかにし

た。また、同海域では体長7mm～90mmの断続的体長組成を持つ稚幼魚が採集されることから、同海域のブリ稚幼魚はかなり南方水域で発生して黒潮に流されたものと、伊豆方面でふ化したと推定されるものが混在して来遊すると推測した。さらに小達 (1967) は、1949～1966年の間の表層稚魚網の採集記録から、ブリは6月に最も多く採集されることを明らかにした。1963年には農林水産技術会議の特別研究「モジャコ採捕のブリ資源に及ぼす影響に関する研究」が開始されて、ブリの卵仔稚魚が多数採集され、対馬暖流域に比べて情報が乏しかった太平洋沿岸においても産卵海域や産卵期に関する具体的資料が充実し(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967), ブリの産卵は東シナ海中南部で2～3月、九州・四国近海で3～5月に行われ、季節が進むに従って産卵の重心が北上し、6月には四国東部近海で産卵場が形成されるとした。Safran (1990) と Safran and Omori (1990) は、小達 (1962, 1967) 以降の三陸水域の情報を補足した。花岡 (1995) は、「モジャコ採捕のブリ資源に及ぼす影響に関する研究」以降も太平洋岸で継続してモニタリングしてきたモジャコ漁場一斉調査の資料を収集して整理した。また、石田ら (1997) は、1978～1995年の間に九州西岸・薩南海域から紀南海域にかけて行われた表層曳き稚魚網を用いた調査資料を整理して、当海域におけるブリ仔稚魚の分布資料を充実させた。その結果、九州西岸から三陸水域の仔稚魚の季節的分布の変遷が明らかになった。

成熟期にある親魚の地理的分布と出現時期 木村 (1952) は、1940～1945年までの6年間、九州沿岸から日本海側では島根県、太平洋側では外房沿岸までのブリ生殖腺の成熟状況について調べ、大型の個体ほど成熟が早いという傾向を見だし、2月に九州南部海域で産卵が始まり、徐々に東方へ産卵場が移っていくことを推察した。また、熊野灘から伊豆半島の沖合に産卵場が形成されることを推察した。これまでブリでは天然魚の生殖腺の組織学的観察は行われていない。三谷 (1960) は、卵巣及び卵巣卵の形態を観察し、発達程度によって5つの成長段階に分けたが、この中の熟度V(完熟期)は、その記述から吸水卵を持つ個体と判断出来る。そこで、完熟期に当たる個体は、採集海域で産卵するものとして本項では取り扱う。また、三谷 (1960) は、生殖腺指数(G_I ; $G_I = \text{生殖腺重量} \times 10^4 / \text{尾叉長}^3$)と卵巣の成熟段階の対応をみて、完熟期にあるものは $G_I > 15.3$ と言う関係を明らかに

*1 山本敏博, 佐々千由紀, 小西芳信, 2005: 東シナ海におけるブリ属仔稚魚の表層分布と成長, 2005年度日本水産学会大会講演要旨集, 32.

*2 山本敏博, 佐々千由紀, 小西芳信, 2005: 東シナ海におけるブリ属卵仔稚魚の分布, 2005年度日本水産学会大会講演要旨集, 32.

した。三谷 (1960) は、1958年4、5月に男女群島で採集した雌親魚、1959年5月に島根県浜田沖で採集した雌親魚、1959年5月に若狭湾で採集した雌親魚、さらに1957年4月と1959年4、5月に熊野灘で採集した雌親魚の卵巣の熟度と G_1 をみることによって、男女群島(4、5月)と熊野灘(4月)での産卵を確認した。一方で三谷 (1960) は島根県沿岸、若狭湾では生殖腺が完熟期に値する生殖腺を持つ個体を得ていないが、産卵の可能性が高いことを報告した。村山 (1992) は、東シナ海の陸棚内縁上で3~5月に漁獲されたブリの雌親魚生殖腺の G_1 をみることによって、漁獲されたものの一部は産卵群であることを確認した。辻 (2000) は、1996年5、6、11、12月、1997年5、6月に能登

半島沿岸でブリの雌親魚生殖腺の G_1 をみることによって能登半島周辺で6月以降の夏季に産卵の可能性があると指摘した。しかし、 G_1 は1997年6月に採集した6.33の1個体が最高で、完熟期までにはほど遠く、能登半島周辺での産卵を裏付けるためには更に7、8月のサンプル確保、卵仔稚魚採集が必要である。

卵仔稚魚の地理的分布と出現時期 日本周辺海域を小海域へ区分した図を作成し (Fig. 1), 海域別に卵と仔稚魚の出現時期をまとめた (Table 1)。これまでの報告では発育段階が明確に示されているものが多いので、ここでは発育段階を区別せず、仔稚魚として記述した。卵の採集記録は五島灘から対馬海峡 (D) と薩南海域 (H) に限られ、その時期はそれぞれ4、

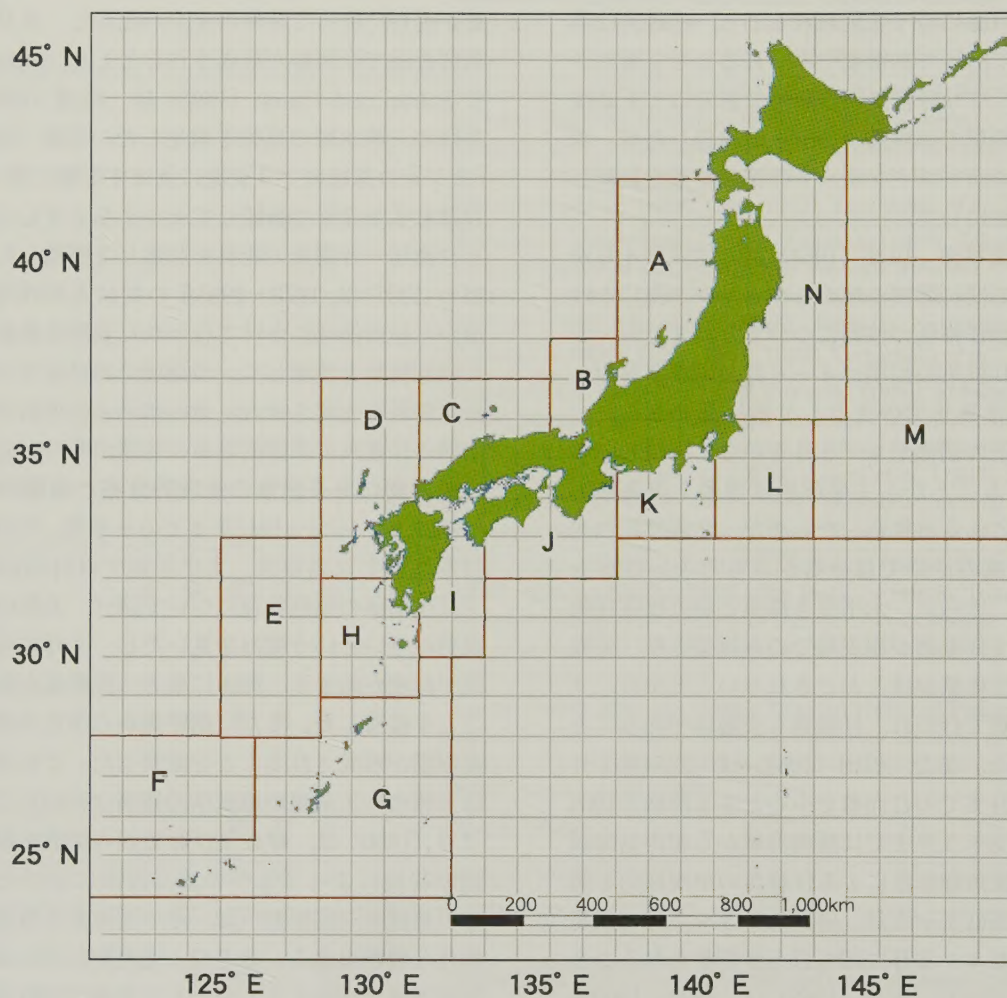


Fig. 1. A sectional map around Japan used in this paper.

A: Northern part of the Sea of Japan; B: From Wakasa Bay to off Noto Peninsula; C: San'in and Oki Islands; D: Around Goto Islands and Tsushima Channel; E: Middle part of the East China Sea; F: Southern part of the East China Sea; G: Around Nansei Archipelago; H: South of Kagoshima Prefecture; I: The Sea of Hyuga; J: South of Shikoku and Kii Peninsula; K: From the Sea of Enshu to Izu Islands; L: off Boso Peninsula; M: Kuroshio extension area; N: From off Johban to off Sanriku.

Table 2. Occurrence frequency of eggs and larvae by water temperature. The notations used for areas are the same as Fig. 1. Upper and lower lines of each area indicates larvae and eggs, respectively. Occurrence frequency; Δ : 1-several, $\bigcirc$: dominant, $\odot$: predominant. Literatures by Uchida *et al.* (1958a, b) referred to eggs as well as larvae.

Area	13°C~	14~15~	16~17~	18~19~	20~21~	22~23~	24~25~	26~	Sources
A					$\odot$	$\odot$	$\odot$	$\odot$	Shimomura and Fukataki (1957), Ikehara (1977)
B		Δ	Δ	Δ	$\odot$	$\odot$	$\odot$	$\odot$	Katoh (1955), Shimomura and Fukataki (1957), Fukataki (1958)
C		Δ	Δ	Δ	$\odot$	$\odot$	$\odot$	$\odot$	Shimomura and Fukataki (1957), Senta (1962)
D			$\odot$	$\odot$	$\odot$	$\odot$	$\odot$	Δ	Shimomura and Fukataki (1957), Uchida <i>et al.</i> (1958a, 1958b), Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Sakakura and Tsukamoto (1997), Cho <i>et al.</i> (2001), Yamamoto <i>et al.</i> ^(*1,*2)
E				Δ	Δ	$\odot$	$\odot$	$\odot$	Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Yamamoto <i>et al.</i> ^(*1,*2)
F					Δ	Δ	Δ	Δ	Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Yamamoto <i>et al.</i> ^(*1,*2)
G					Δ				Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Yamamoto <i>et al.</i> ^(*1,*2)
H		Δ	$\odot$	$\odot$	$\odot$	$\odot$	$\odot$	Δ	Shimomura and Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967), Yamamoto <i>et al.</i> ^(*1,*2)
I		Δ	Δ	Δ	$\odot$	$\odot$	$\odot$	$\odot$	Shimomura and Fukataki (1958), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967)
J				Δ	Δ	$\odot$	$\odot$	Δ	Hattori (1964), Tokaiku National Fisheries Institute (1966), Tokaiku National Fisheries Institute and Nanseikaiku National Fisheries Institute (1970), Asami <i>et al.</i> (1967)
K				Δ	Δ	$\odot$	$\odot$	Δ	Hattori (1964)
L				Δ	Δ	$\odot$	$\odot$	Δ	Hattori (1964)
M									
N	Δ	Δ	Δ	Δ	Δ	$\odot$	Δ	Δ	Odate (1962)

5月、1～5月である。男女群島から五島南方水域 (D) では5月を中心にブリの卵が採集される。卵は、固定の状況によって査定が困難であったり、他のアジ科魚類の卵との判別の難しさから報告された海域が限られる。仔稚魚は2月に東シナ海中南部海域 (E, F) および薩南海域の陸棚側の黒潮外縁域で採集され始める。3月に入ると、対馬海流域では対馬海峡、太平

洋では紀伊水道外域 (J) まで分布する。4月に入ると、対馬海流域では隠岐周辺 (C)、太平洋ではすでに黒潮続流域、常磐沖 (N) にまで分布がみられ、一方黒潮の太平洋側の黒潮外縁域で、調査定点が少ないにせよ、この海域で仔稚魚はみられなくなる。なお、4月には対馬海流域では五島周辺海域 (D)、太平洋では薩南海域から紀伊水道外域で稚魚の出現盛期に入

る。5月に入ると、対馬海流域では4月と変わらず隠岐、山陰沿岸(C)、太平洋では北緯38度以南の三陸沖(N)にまで分布北限が延びるが、東シナ海南部海域(F)では、採集定点が少ないにせよ、仔稚魚はみられなくなる。なお、5月には対馬海流域では五島周辺海域、太平洋では薩南海域から伊豆諸島近海(K)で仔稚魚の出現盛期が終わる。6月に入ると、対馬海流域では若狭湾(B)、太平洋では北緯39度の三陸沖にまで分布北限が延びる。なお、6月には対馬海流域では対馬海峡での出現盛期が終わり、隠岐周辺から若狭湾での出現盛期が始まる。また、太平洋では三陸沖で出現盛期を迎える。7月には、対馬海流域では秋田県男鹿半島北西沖(日本海の分布北限)(A)、太平洋では北緯40度(太平洋の分布北限)を越えて分布するが、東シナ海中部域では仔稚魚がみられなくなる。なお、7月には対馬海流域では五島周辺海域で出現盛期が終わり、太平洋では全域で出現の終期となる。8月以後は、対馬海峡以东の日本海(A~D)での出現が主となり、太平洋では9月の伊豆諸島近海の採集記録のみとなる。

産卵好適水温 Table 2に海域別に卵と仔稚魚の出現水温をまとめた。卵の出現した水温の記録は男女群島から五島南方水域に限られ、その水温は19~22℃台である。仔稚魚の出現は例外的に三陸沖の12℃台から認められ、日本海側では山陰西部海域から能登半島外浦までの海域と、太平洋側での薩南海域から日向灘にかけての海域では14℃台からみられる。九州西岸域から対馬海峡並びに薩南海域では15~22℃台まで出現盛期となり、同海域では他の海域に比べ低い水温から盛期を迎える。山陰西部海域から能登半島外浦にかけての日本海西部海域と、日向灘では18℃台から出現盛期に入る。また、日本海北部海域では20℃台から突然盛期を迎え、一方紀伊水道外域から房総沿岸の太平洋岸では18℃台から出現し始め、20℃台から盛期を迎える。山陰西部海域から能登半島外浦の日本海西部海域と紀伊水道外域から房総沿岸の太平洋では23℃台まで出現の盛期があり、その後26℃台、場合によっては29℃台まで出現が認められる。東シナ海南部海域では最低でも20℃台から出現し始め26℃台まで出現する。東シナ海中部海域では17℃台から出現を始め19~22℃台まで出現盛期で、その後25℃台まで出現がみられる。分布の末端の三陸沖では21℃台まで出現がみられ、19℃台で出現盛期となる。今回の整理では、三陸沖と記録の無いまたは少ない東シナ海中部海域と黒潮統流域を除く海域で、20~22℃台に仔稚魚の出現好適水温帯が

みられたことから、産卵好適水温帯は三谷(1960)の19~21℃と考えても良いと思われる。

産卵海域と産卵期の推定 Fig. 2に産卵海域と産卵期についてまとめて記す。東シナ海中部海域におけるブリ卵の採集記録は無いが、当海域で2月に稚魚が採集されていることから2月には産卵が始まることは間違いないと考えられる。また、当海域における産卵水温は、20℃台以上である。台湾北部沿岸の表層最低水温^{*3}が約20℃(1, 2月)であることから、当海域では最も早い産卵が1月には始まると考えられる。また、薩南海域で1月に2個のブリ卵採集記録がある(松田, 1969)のは例外としても、2月は東シナ海中部海域を中心として、最も北では薩南海域でも産卵が行われている可能性があると考えられる。3月は卵の採集記録や親魚の成熟状況から、対馬海流域では男女群島以南と、太平洋では薩南以西の海域で産卵が行われていると考えられ、産卵の中心は東シナ海中部海域・クチミノセ近隣海域と推定される。さらに、4月は対馬海流域では対馬・壱岐以西の海域、太平洋では熊野灘以西の海域で産卵が行われていると考えられ、産卵の中心は東シナ海中部海域から男女群島並びに薩南海域と推定される。なお、4月に東シナ海南部海域でも稚魚が採集されていることから(山本ら^{*1})、当海域でも産卵が継続して行われていると考えられる。5月は対馬海流域では山口県見島以西の海域(山本ら未発表)、太平洋では伊豆諸島以西の海域で産卵が行われていると考えられ、産卵の中心は対馬暖流域では男女群島から対馬、壱岐にかけての島嶼沿岸、太平洋では薩南海域から伊豆諸島にかけての島嶼沿岸と推察される。なお、太平洋側での産卵は伊豆諸島近海が東限と考えられる。また、東シナ海中部海域では5月には産卵はほぼ終了すると推定される。6月は卵の採集記録が無いが、10mm未満の稚魚の採集記録と親魚の成熟状況から、太平洋では各海域で産卵が終期に入る。一方、対馬暖流域では五島列島から山口県見島で産卵が終期を迎え、隠岐周辺海域から若狭湾へ産卵海域が移ると推定される。その後、7~8月にはその中心が能登半島沿岸へ移動してブリの産卵は終了すると推定される。

産卵海域と産卵期に関する知見は、親魚の成熟状況調査や卵仔稚魚の採集調査によってかなりの点で充実した。しかし、産卵親魚の排卵後濾胞の形成過程を組織学的に確認し、漁獲された天然魚の排卵後濾胞を組織学的に確認することによって、産卵時間や産卵海域を絞り込む必要があるなどの課題も多い。

^{*3} 独立行政法人水産総合研究センター、水産海洋データベース (http://jfdb.dc.affrc.go.jp/kaiyodb_pub/system/download1.html)

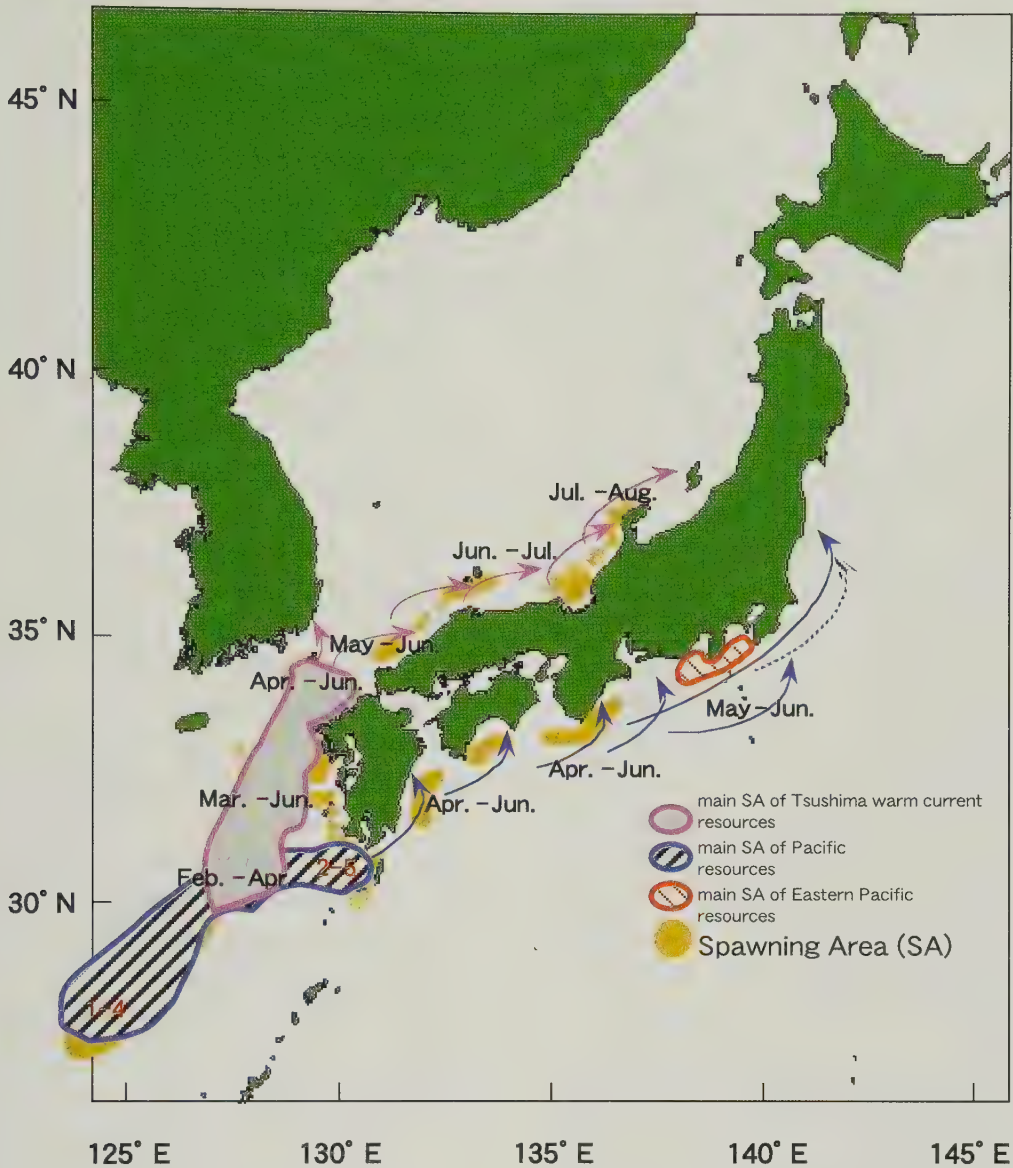


Fig. 2. Spawning month in respective areas of yellowtail estimated from past studies.

2-2 卵仔稚魚の発生と成長

ブリの卵発生および仔稚魚の形態については内田ら (1958a, b) が詳細に報告している。それによると卵は分離浮性卵で、直径は1.19~1.27mm (平均1.25mm), 卵黄内に油球 (径0.30~0.33mm) が1個ある。水温18~24℃で51時間後にふ化する (ふ化後3日までの仔魚の形態記述は省略)。しかし、ふ化仔魚の3日以上飼育は成功しておらず、仔稚魚の形態は天然海域から得られた断続的な成長段階の標本に基づき記載された。Sakakura and Tsukamoto (1996) はふ化仔魚を用いて耳石の日周輪形成を確認し、さらに五島列島北西海域で採集した稚幼魚の日齢解析を行い、同海域における成長速度を明らかにした。上原ら (1996) と

山本ら^{*1}も耳石日輪解析を行って成長速度を明らかにした。Fig. 3にこれらの成長解析結果を示す。なお、村山 (1992) は飼育した仔稚魚と飼育日数の関係を成長式としているため、個体ごとの耳石日齢解析結果とは異なる。成長式は、報告者によって全長、尾叉長、標準体長が用いられているため単純な比較は出来ないが、成長速度はそれぞれで異なる。特に上原ら (1996) と山本ら^{*1}の成長式を比較すると約1.8倍の速度差が認められた。上原らは1995年5月に土佐湾で採集された標本を用いているのに対し、山本らは2002年4、5月と2003年4月に東シナ海で採集された標本を用いている。土佐湾に5月に来遊するモジャコは東シナ海または隣接する薩南海域を起源とするものが多い (村

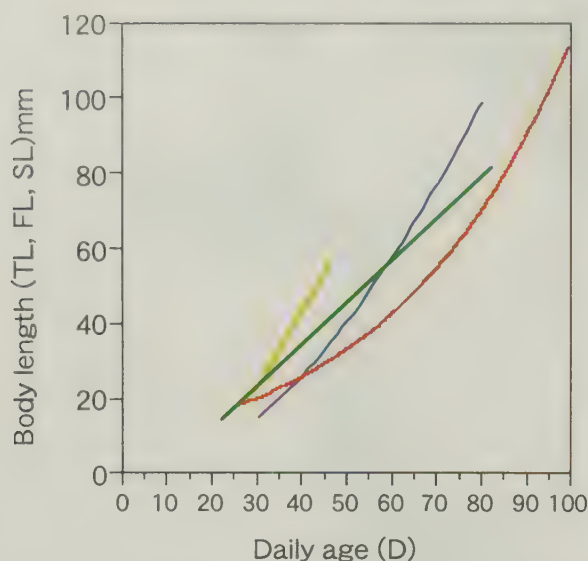


Fig. 3. Growth curves of yellowtail obtained from rearing experiments and otolith daily increment analysis.

Blue line: $TL = 4.52 - 3.23 \times 10^{-1}D + 2.49 \times 10^{-2}D^2 - 7.56 \times 10^{-5}D^3$ (Murayama, 1992).

Red line: $TL = 9.61 \times e^{(0.025D)}$ (Range: 18.1–113.8mm. Sakakura and Tsukamoto, 1997).

Yellow line: $FL = 2.029D - 38.368$ (Range: 20.72–56.08mm. Uehara *et al.* 1996).

Green line: $SL = 1.127D - 11.012$ (Range: 15.1–81.7mm. Yamamoto *et al.* *¹).

TL: Total length. FL: fork length, SL: standard length. D: days after hatching.

山, 1992; 東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970) ことを考えると両報告の稚幼魚の起源はほぼ同じと考えられ, 成長差は成長過程で経過した物理環境や餌料環境の違いによって生じた可能性が考えられる。

2-3 仔稚幼魚の加入と移動

日本沿岸へ来遊する魚類仔稚の輸送と流れ藻との関わりは古くから注目され, 特にブリやサンマは初期生活史と流れ藻の関係が深い魚類として知られてきた(例えば, 内田, 庄島, 1958; 内田, 1955)。吉田 (1963) は, 日本周辺各水域の流れ藻の分布について広域的に調べ, またその季節量変化と移動について漂流はがき, 漂流瓶, 流れ藻への標識調査によって明らかにし, 主に流れ藻の藻類学的知見を総括した。千田 (1965) は, 流れ藻と魚類仔稚の関係について論じ, 流れ藻の水産的効用について総括した。次いで, 東海区水産研究所 (1966), 東海区水産研究所・南西海区水産研究所 (1970), 能津ら (1967), 田中 (1967) は, 「モジャ

コ採捕のブリ資源に及ぼす影響に関する調査」の中でブリ稚幼魚と流れ藻の関係について総括的研究を行った。その中では, ブリ幼魚の標識放流, 流れ藻の標識放流等を組織的に行い, 主に太平洋岸における流れ藻の移動速度や寿命, さらににはブリの沿岸への加入機構を論じた。ところで, ブリの稚魚は体長約15mmまで浮遊生活を送り, その後, 体表への横縞の発現と同時に流れ藻への随伴性が発現することが確かめられている(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967; 山本ら*¹)。しかし, 体長15mmに達するまでの卵仔稚魚の生活史に関する情報は少ないのが現状である。

流れ藻の寿命は2週間～1ヵ月が平均的であることが明らかになっている(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967; 千田, 1965)。また, 流れ藻の移動速度は日本海・対馬暖流と太平洋・黒潮ではそれぞれ, 6～11海里/day, 10海里/dayであるが(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967; 吉田, 1963; 千田, 1965), 太平洋では最も移動の速いもので40海里/dayを越えるものもあり(東海区水産研究所, 南西海区水産研究所, 1970), これは黒潮流軸に取り込まれた場合と考えられる。流れ藻の移動速度とブリ幼魚の移動速度, 流れ藻の寿命から考えて, 流れ藻に随伴するブリ稚幼魚は数日ごとに近隣の流れ藻へ移りつつ, 流れ藻とともに沿岸へ来遊するものと考えられる(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967)。しかし, 中には流れ藻に随伴しない稚幼魚の存在も指摘されており(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967), この点の解明は今後の課題である。

流れ藻は主に沿岸域に分布し, 沖合域にはほとんど分布しないことが報告されている(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 吉田, 1963; 千田, 1965)。このことから主産卵場のある東シナ海で発生したブリ仔稚魚は九州西岸域から対馬海峡並びに薩南海域の沿岸で流れ藻に随伴して日本海および太平洋の沿岸へ流れ藻とともに加入すると考えられてきた(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967)。しかし, 小西 (2000) は, 2000年4月に東シナ海の陸棚沖合域で流れ藻が多く分布していることを確認し, この流れ藻が中国大陸沿岸から流れてくる可能性を指摘した。同時に, ブリ仔稚魚は東シナ海の沖合域で流れ藻と出会って, 沿岸域へ加入する可能性を

報告した。今後は東シナ海沖合域の流れ藻の調査も重要と考えられる。

2-4 食性

安楽, 畔田 (1965) は、九州西岸域から薩南海域にかけて採集されたブリ仔稚幼魚の食性を明らかにした。流れ藻に着く以前の稚魚は小型カイ脚類を主とする枝角類等の動物プランクトンを食しており、その後、全長27mm前後で魚食性が発現する。全長80mmで動物プランクトンと魚類の摂餌割合は等しくなり、全長130mmで完全に魚食性に移行することを報告した。また、5月中旬以降に採集されたブリ稚幼魚の共食いについて報告している。しかし、同じ大きさでも(わずかに)海域の異なる群れでは、完全に動物プランクトン食の群れと魚食の割合の多い群れがあることに触れ、海洋の物理・化学環境や餌料環境、摂餌時間帯による差の可能性を推察している。そして、ブリ稚幼魚の流れ藻への随伴性と、流れ藻による餌料供給の関係に触れてそれを否定し、ブリ稚幼魚の流れ藻への随伴性は逃避行動と関係があると推察した。一方、千田 (1962b) は、隠岐周辺で採集した流れ藻に随伴するブリ稚幼魚の食性を明らかにし、それらは流れ藻に産み着けられたサンマの卵を飽食していたことから、ブリは流れ藻を主に索餌の場として利用していると推察した。「モジャコ採捕のブリ資源に及ぼす影響に関する研究」(東海区水産研究所, 1966; 東海区水産研究所, 南西海区水産研究所, 1970; 浅見ら, 1967; 能津ら, 1967; 田中, 1967) では、モジャコの採捕が漁獲加入資源へ与える影響を評価するために、初期減耗要因の解明が課題として上げられ、主にブリの共食いに関する知見が集積された。ブリの共食いは海域によって違いがみられ、共食い出現率は1965年の太平洋南岸の全域平均で5.39%, 九州西海域で1963年は0.5%, 1964, 1965年は0%であったことを報告している(東海区水産研究所, 1966)。その差の理由として、太平洋南海域ではモジャコの発生時期が進むにつれて大きさの異なる個体が群れを形成し共食いを誘発するが、九州西岸では比較的大きさの整った個体が群れを形成しているために共食いの誘発は抑えられると推察した(東海区水産研究所, 1966)。Sakakura and Tsukamoto (1996) は、1992年5月に五島列島西岸海域で採集した流れ藻に随伴するブリ稚幼魚を採集し、東海区水産研究所 (1966) の調査では共食いの見られなかった海域での共食いを確認し、共食いの起こる群れは複数のモードを持つ群れから形成されており、大型の群れの個体が小型の群れの個体を共食いしていることに触れ、共食いは群れの構成員を選択するための

行動と推察した。安楽, 畔田 (1965) が報告した共食いの有無も同一群内におけるサイズ組成に依存するということで説明できると考えられる。また、共食いによる減耗率の試算では、1,000尾のモジャコが1月後には335(最小)~55(最大)に減少すると試算した(東海区水産研究所, 1966)。Sakakura and Tsukamoto (1996) は、人工ふ化させたブリ仔魚を用いて、共食いの発現時期を実験的に明らかにした。共食いは、ふ化後22日まで(9.6mmTL)はみられなかったが、23日目から発現したことを報告している。ふ化後22-23日齢は、仔魚から稚魚へ変態する時期と重なっており、その後共食いは39日齢目まで発達した。しかし、途中の33-36日齢では一旦共食いが減少しており、33日齢目には群れ形成が確認され、個体間距離の確保が図られる行動を報告した。また、共食いの捕食個体(X_c)と被食個体(Y_p)の全長の関係を解析し、 $Y_p = 0.49X_c + 0.30$ ($r = 0.97$) の関係式を導いた。

Kohbara *et al.* (2003) は、50gと80gの幼魚を飼育して、水温と光に対する自発摂餌を明らかにした。ブリ幼魚は18℃以下の水温では自発摂餌は低下するが、18℃を越えると高くなり、その後水温を上昇させてもその活性は頭打ちとなった。また、光に対するその活性は薄暗い時間帯から夜間に高くなり、ピークは夜明け前と夕暮れ時の二度みられることを報告した。

以上のようにブリ仔稚幼魚の初期減耗の要因の一つに、食性に関わっていることが示唆された。しかし、浮遊期仔稚魚の餌料生物については不明な点が多く、天然海域における初期餌料の解明が必要と考えられる。また、仔稚魚期の自発摂餌と水温、日周リズムの関係については実験的な検討が必要と考えられる。

第3章 移動・回遊に関する既往知見

3-1 対馬暖流域におけるブリの分布・移動・回遊 年齢別の分布・移動・回遊

対馬暖流域におけるブリの移動・回遊については、渡辺 (1979) が、主に1960年代以前に行われた対馬暖流域における標識放流結果をとりまとめて報告しているほか、永田 (1959)、三谷 (1960)、沢田ら (1960) などの報告がある。それらを総合すると、対馬暖流に輸送されて日本海へ入った0歳魚は、北海道沿岸から極前線まで(山川, 1989)の日本海の広い範囲に分布し、秋冬季の水温降下に伴い南下して、佐渡海峡以南で越冬する(Fig. 4)。1, 2歳魚は大規模な回遊をせず、春から夏の水温上昇期に北上し、秋から冬の水温下降期に南下するという季節的な小規模回遊や沿岸から沖合へと深浅回遊を繰り返す。そして、各海域の湾、島嶼、浅瀬、礁付近

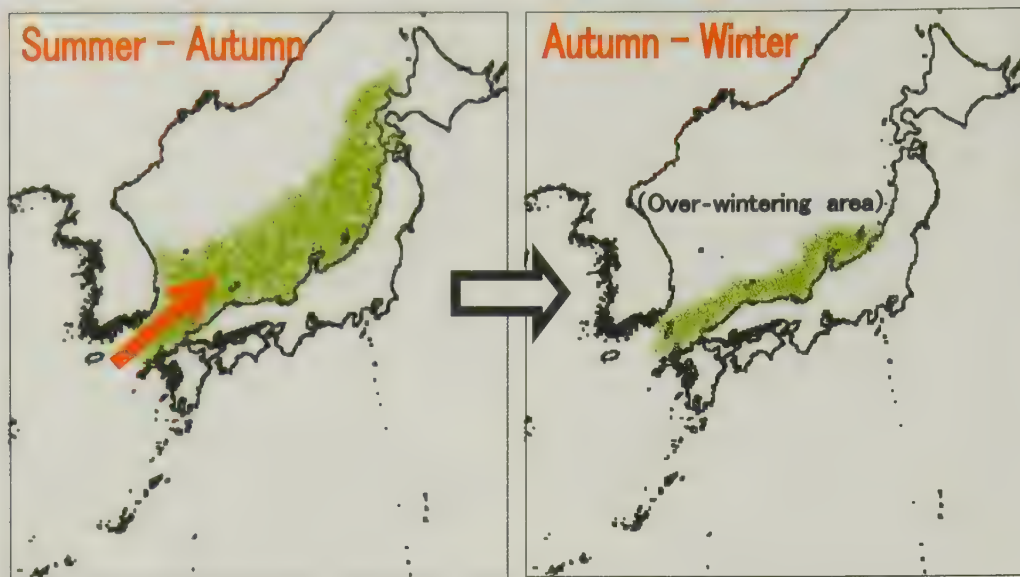


Fig. 4. A schematic diagram on distribution and migration of 0-year old yellowtail in the Sea of Japan before the 1960s (after Watanabe, 1979; Yamakawa, 1989 and Ino *et al.*, 2006).

に長期間滞留し、活発な索餌行動を行うが、1歳に比べて2歳の移動範囲が広い。

尾叉長19.0cm以下の0歳魚を、山陰沖で標識放流した結果では、再捕位置はすべて放流点よりも東の沿岸域であり、隠岐島よりも沖合側で放流された個体は富山湾以東で再捕され、本州寄りの海域で放流された個体は能登半島以西で再捕されている(村山, 1992)。稚魚は、流れ藻に付随し、海流に伴って移動するため、その移動は対馬暖流の流れの影響を大きく受けるものと推測される。流れ藻を離れ、沿岸域に加入来遊した、尾叉長21.0~40.0cmの0歳魚を標識放流した結果では、例えば富山湾で放流された個体は、殆どが富山湾内で再捕されるなど、再捕海域は放流海域およびその隣接海域に限られており(村山, 1992)、流れ藻に付随する時期と比較して移動範囲が狭い。

1歳魚の標識放流結果から渡辺(1979)は、1歳魚は移動範囲が狭く、放流点から30海里以内の再捕が圧倒的に多いとしている。村山(1992)および一丸ら(1993)によれば、1歳魚を長崎県対馬付近で標識放流した結果では、再捕位置は山陰沖から五島列島の範囲であり、大部分は放流海域付近で再捕されている。2歳魚についても渡辺(1979)は、移動範囲が狭く、山口県と島根県、若狭湾と能登西岸というように隣接海域との関連が強いとしている。また、3歳魚では、南下期に男鹿半島から北陸沿岸へと大きく移動する個体がみられ、南下期に移動範囲が拡大するとしている。

3歳の南下期以降は、生活周期が越冬期から産卵期

へと連続して移行していくために回遊範囲が広がり、本格的な季節回遊を開始する。4歳以上では、北海道沿岸から東シナ海におよぶ間を南北に回遊するようになるものと推測される。井野ら(未発表)は、1999年からアーカイバルタグ(記録型標識)を使用した標識放流調査を行い、4歳以上のブリが北海道沿岸と主産卵場である東シナ海を南北に往復回遊することを確認した。

4歳魚以上では、北海道沿岸と主産卵場である東シナ海を南北に往復回遊する個体のほか、能登半島あるいは山陰沖と東シナ海を南北回遊する個体、および1年間以上も能登半島周辺に滞留する個体が存在することが確認されている(井野ら, 未発表)。

以上を整理すると、ブリは成長段階に応じて回遊様式が変わり、0歳魚として受動的な輸送を経て、各地の沿岸域に加入した後、2歳まで小規模な回遊を行いながら成長し、3歳の南下期以降は北海道沿岸から東シナ海の大陸棚縁辺部におよぶ間を南北に往復回遊するものと推測される(井野ら, 2006)。

年代別の分布・移動・回遊 年代によってブリの分布域および移動・回遊のパターンに大きな違いが見出されることが示されているほか、それに伴うとみられる漁況の変動が生じたことも報告されている。原(1990b)は、1971~1986年の対馬から佐渡島にいたる日本海沿岸域の定置網漁業における銘柄別の漁獲量の変動から、1960年代以前と比較して、1970年代から1980年代にわたり、成魚の分布域が縮小するとともに若齢魚の分布域が西偏したことを示唆した。

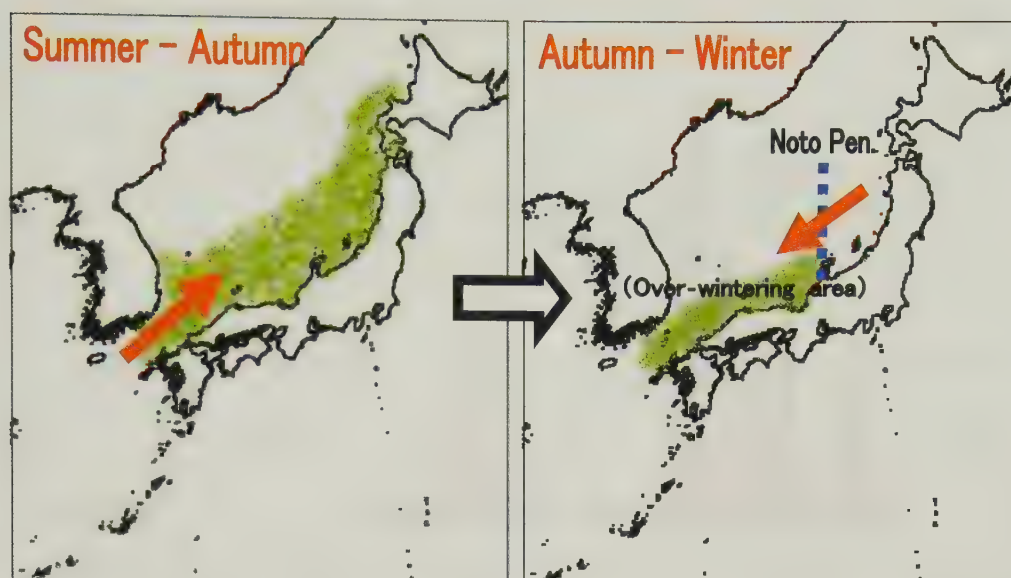


Fig. 5. A schematic diagram on distribution and migration of 0-year old yellowtail in the Sea of Japan during the 1970s and 1980s (after Watanabe, 1979; Yamakawa, 1989; Murayama, 1992 and Ino *et al.*, 2006).

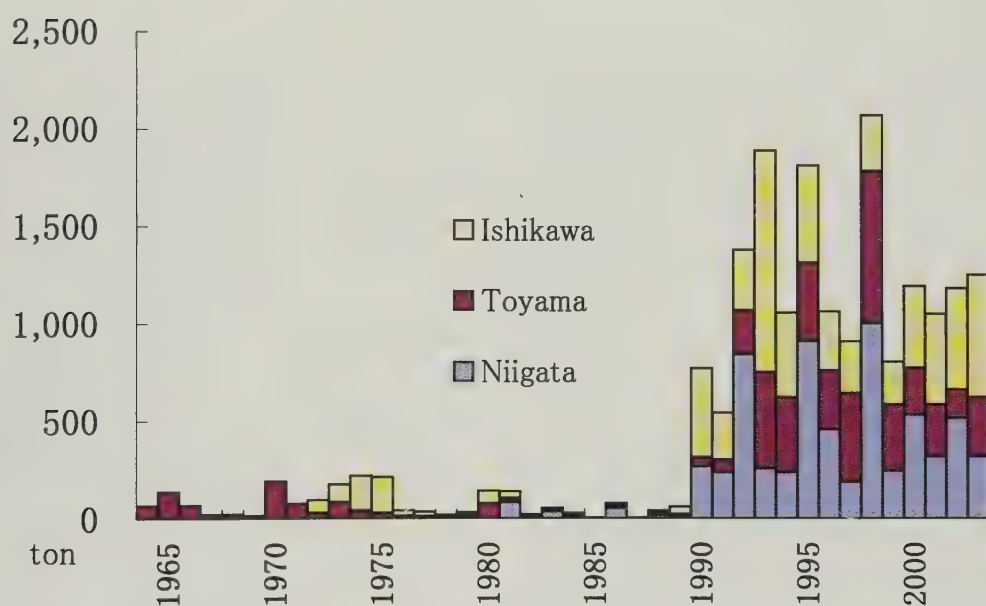


Fig. 6. Yearly catch amount of yellowtail over 2-years old caught by fixed nets in Niigata (from 1981 to 2003), Toyama (from 1964 to 2003) and Ishikawa (from 1972 to 2003) prefectures (after surveys conducted by each prefectural experimental institute).

村山（1992）は、1950年代以前と1960～1980年代の標識放流結果を比較検討し、1950～1960年代を境にブリの回遊様式が変化したことを見出した。1950年代以前は、能登半島以北つまり日本海北部の沿岸域に來遊した0歳魚が、日本海北部で越冬したが、1970～1980年代は日本海北部で越冬せずに能登半島を越えて南下し（Fig. 5）、成長後も能登半島以北へ回遊せず、北海道

と東シナ海を南北に回遊する大型魚が減少したことを報告している。1970～1980年代には、富山湾など日本海北部の定置網で大型魚（2歳以上）の漁獲量が低迷したが（Fig. 6）、これは、未成魚期からの分布・回遊様式の変化に伴い、日本海北部において1歳以上のブリの分布量が減少するとともに、北海道と東シナ海を南北に大回遊する個体が減少し（村山、1992）、日

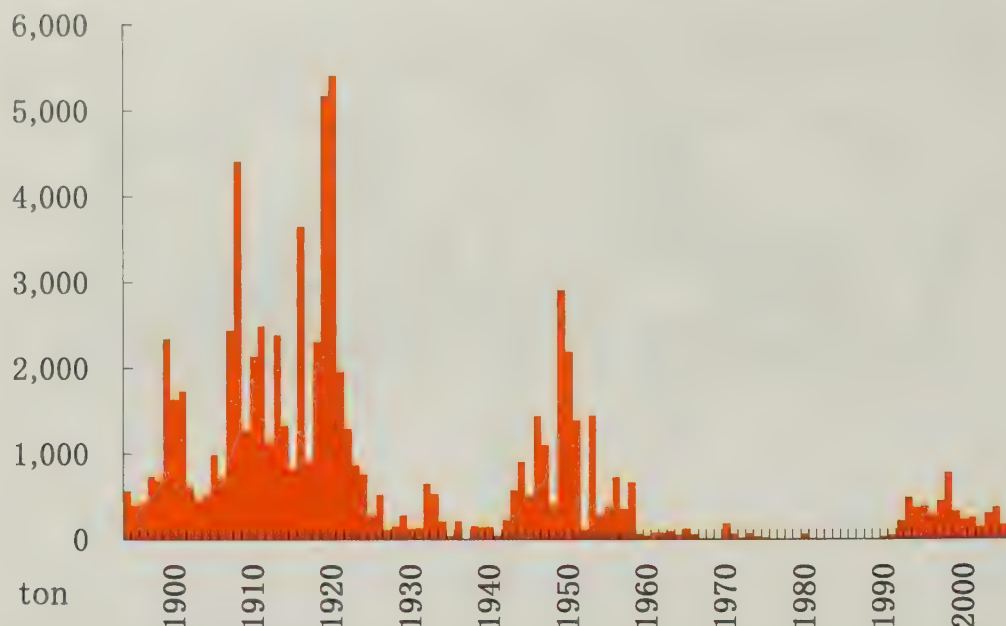


Fig. 7. Yearly catch amount of yellowtail over 2-years old by fixed net in Toyama Prefecture (after surveys conducted by Toyama Prefectural Experimental Institute and catch statistics compiled by the Ministry of Agriculture Forestry and Fisheries).

本海北部の定置網漁場へ来遊する大型魚が減少したことに伴うものと推測される（井野ら，2006）。一方，1990年代には日本海北部の定置網で大型魚の漁獲量が増大した。1990年代にも分布・回遊様式が変化したものとみられ，北海道と東シナ海を南北に大回遊する個体が増加したことによって漁況が変化したものと推測される（井野ら，2006）。

三谷（1960）は，主要な道府県におけるブリの漁獲量について解析し，漁獲量の動向には東シナ海区（福岡県～鹿児島県）と日本海北区（青森県～石川県）で相反する傾向があり，二十数年周期で変動していると指摘している。大型魚の漁況変動について，伊東（1959）は，1490年代から1940年代の丹後伊根浦（京都府伊根町）をはじめとする日本海沿岸各地（北海道～山口県，長崎県）の漁獲量について調査し，各地において，定置網漁獲量の豊凶に周期的変動がみられることを示している。さらに，富山県の定置網における大型魚の漁獲量（Fig. 7）をみると，数十年周期で漁獲量の豊凶がみられる（井野ら，2006）。

分布・移動・回遊と海洋環境 分布・回遊様式の変動は，海洋環境の変化と対応する可能性があることが報告されている。内山（1997）は，1981～1993年の対馬暖流域における銘柄別漁獲量を調べ，資源水準が大きな変化をしていないと述べるとともに，1980年代後半から富山湾の水深50m層における最低水温期（3，4月）の水温が高めに推移していることを見出した。

そして，富山湾の水温変化の特徴は対馬暖流域沿岸の水温変化の特徴を反映したものであるとした上で，1990年代の日本海北部における大型魚（2歳以上）の漁獲量の増大は，資源の増大を反映したものではなく，分布・回遊様式の変化と関連している可能性があることを指摘した。富山湾内17定点の水深50m層における3，4月の平均水温と富山県の定置網における大型魚の漁獲量（Fig. 8）をみると，水温上昇と漁獲量の増大が対応していることがわかる（井野ら，2006）。井野ら（未発表）は，アーカイバルタグを使用した標識放流調査を行い，その結果，3，4月の最低水温期に，富山湾および能登半島付近の範囲を遊泳していた標識魚（3歳および4歳）が見出された。その期間における遊泳層水温（50m層）のデータを調べた結果，標識魚が9℃以上の海域を遊泳していたことがわかった。越冬海域の形成には，最低水温期の水温が大きく影響するものと推測され，水温9℃以上の海域ならば越冬が可能であるとみられた。したがって，最低水温期（3，4月）における，水温9℃以上の海域の分布範囲が越冬海域の範囲を左右する可能性があると考えられる。1980年代と1990年代の3～4月の水温分布を比較すると（Fig. 9），1990年代は日本海北部に9℃以上の海域が拡大した傾向がみられ，これが1990年代を境にしたブリの分布・回遊様式の変動要因の一つである可能性がある（井野ら，2006）。

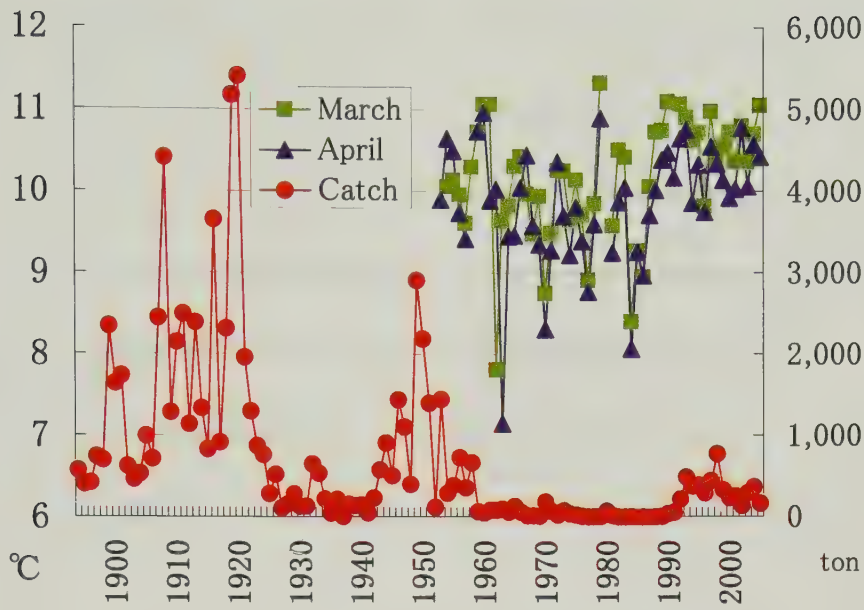


Fig. 8. Average water temperatures at 50m depth at 17 stations in Toyama Bay in March and April, and catch amount of yellowtail over 2-years old caught by fixed nets in Toyama Prefecture.

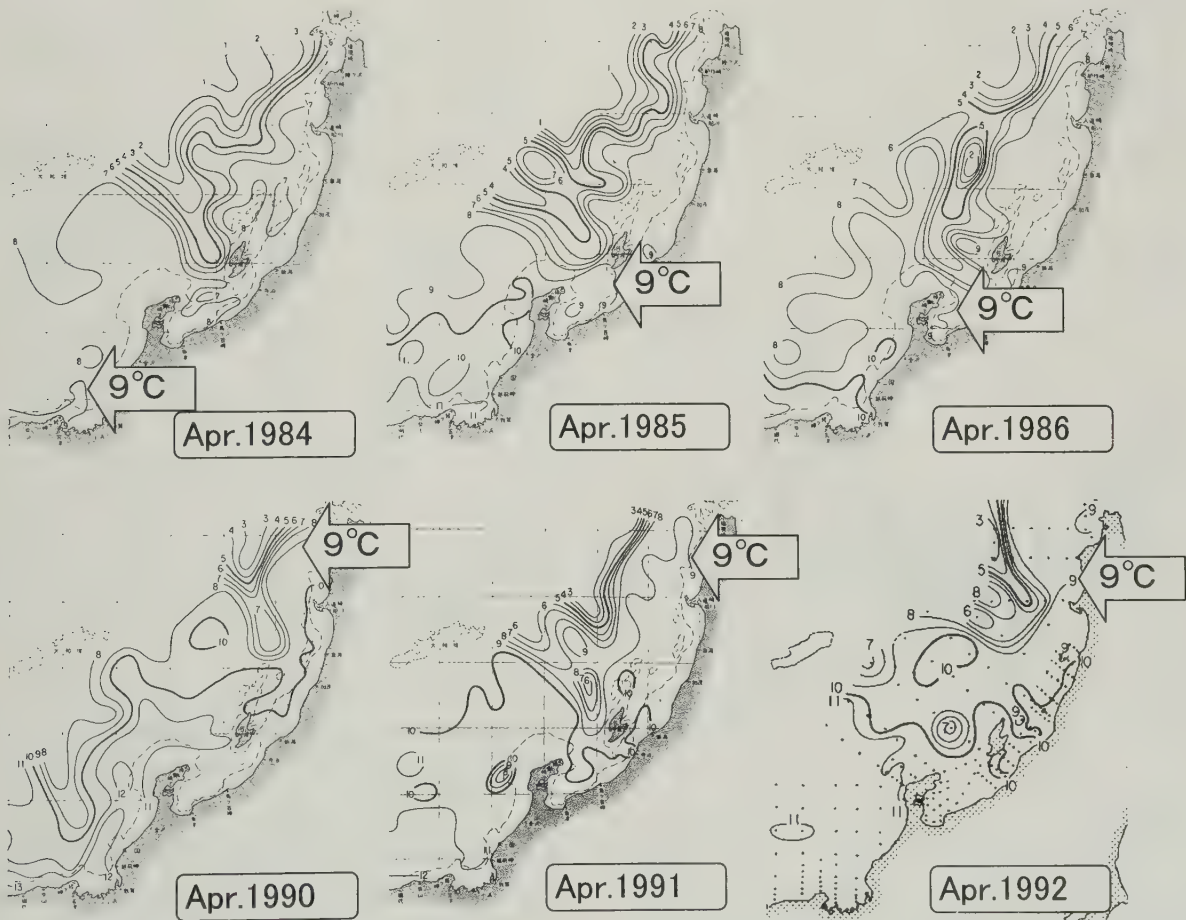


Fig. 9. Contour maps of water temperatures at 50m depth in the Sea of Japan in April, from 1984 to 1986 and from 1990 to 1992 (after Nihon-Kai Gyojyo-Kaikyo Sokuho (Japan Sea National Fisheries Research Institute)).

3-2 太平洋沿岸におけるブリの分布・移動・回遊
年齢別の分布・移動・回遊 太平洋沿岸におけるブリの分布・回遊については、田中（1972a, b, 1973）、Tanaka（1979, 1984）が、1960年代までに行われた標識放流結果および各海域における体長組成の推移等を取りまとめて報告している。また、1980～1990年代に日本栽培漁業協会が静岡県、三重県、和歌山県、徳島県および高知県に委託して実施した「天然ブリ仔資源保護培養のための調査実験」および「ブリ種苗放流技術開発調査」において0歳魚を中心に標識放流が数多く行われ、1987～1997年の結果が日本栽培漁業協会（1999）等に記されている。それらを総合すると、太平洋沿岸の小型魚（0, 1歳）のうち、東北地方の群は三陸沿岸から房総半島の間を北上・南下回遊するが、相模湾以西の群は大きな移動を示さず、両群は3歳になるまで交流しないと推測される（Fig. 10）。1987～1997年に高知県～静岡県で実施された0歳魚の標識放流結果では、7月に熊野灘で放流された人工種苗魚が10月に岩手県で再捕された例など長距離移動を示した事例がごくわずかにみられるが、放流海域付近での再捕がほとんどを占めており、この海域に加入した0歳魚は大きく移動しないことがこの結果からも推測される。

1960年代までに行われた標識放流結果では、大型魚（2歳以上）は3歳になり成熟に達すると東北地方の

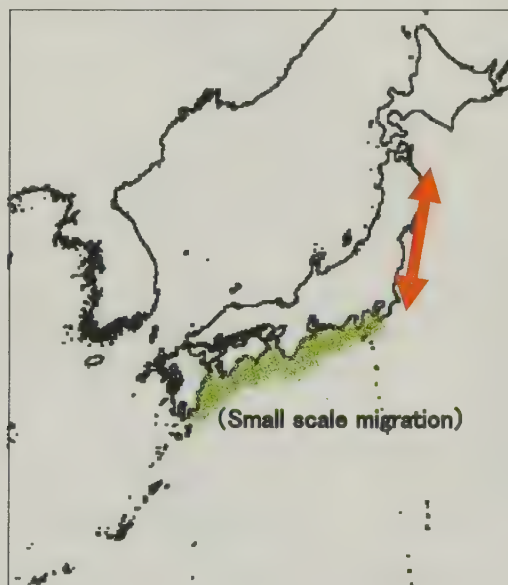


Fig. 10. A schematic diagram on the distribution and migration of 0 and 1-year old yellowtail in the Pacific coast of Japan (after Tanaka, 1972a, b, and 1973).

群と相模湾以西の群れが混合するようになり、三陸沿岸から房総半島を経て相模湾・熊野灘に回遊する群、熊野灘周辺と四国・九州を回遊する群が存在すると報告されている。近年の調査では、1990～1995年に和歌山県で標識放流した大型魚が三重県および四国へ移動した事例が確認されているのみで、遠州灘以東へ移動した標識魚はない。2004年3月、2005年2月および3月には、熊野灘北部で大型魚の標識放流が実施され、和歌山県および四国へ移動した標識魚は確認されたが、房総半島を経て東北地方へ北上した標識魚は2006年3月現在みられていない（久野、阪地、未発表）。

これまでの調査結果から太平洋沿岸においても、0～2歳魚は大規模な回遊をしないものとみられる。3歳魚以上では、三陸沿岸から房総半島を経て相模湾・熊野灘に回遊する群、熊野灘周辺と四国・九州を回遊する群の存在が示唆されるが、近年の標識放流記録が少ないため、詳細は不明である。また、三陸沿岸に来遊する当歳魚についてはこれまでに詳しい調査が実施されておらず、太平洋側を北上した群と日本海側から津軽海峡を越えて来遊した群がどのように分布しているか不明である。さらに太平洋側を北上した群と日本海から津軽海峡を経て太平洋側へ来遊した群がどのように南下するか等の詳しいことは分かっていない。

年代別の分布・移動・回遊 田中（1972a, b, 1973）によると、1950年代以前は東北地方の群および相模湾～潮岬の群が三陸沿岸から熊野灘の間を北上・南下回遊し、潮岬以西の群は東海地方から九州を回遊していたが、1960年代には、東北地方の群および相模湾～潮岬の群が三陸沿岸から相模湾の間を北上・南下回遊するように変化し、東北地方の群および相模湾～潮岬の群の回遊範囲が小さくなったとされる（Fig. 11）。Tanaka（1979）は、放流年に再捕された大型魚の移動について、戦前には潮岬および足摺岬に2つの境界があり、これを越えて南下したものはわずかであったのに対し、戦後は潮岬の境界がなくなり、足摺岬の境界のみとなったとしている。これらの年代による移動・回遊の変化が海洋環境の変化によるものかは明らかにされていない。

分布・移動・回遊と海洋環境 児玉（2003）は、金華山近海域における冬季の気温および春季の水温の長期変動と宮城県における主要魚種の水揚量推移を検討し、ブリ類は暖水年に豊漁、冷水年に不漁の傾向があることを示した。さらに、暖水年にはブリ類の分布域が三陸まで広がり、冷水年には常磐以南へ縮小し宮城県沿岸におけるブリ0歳魚の漁獲量が極めて少なくなるとしている。

ブリ成魚の漁獲量（Fig. 12）は太平洋沿岸において

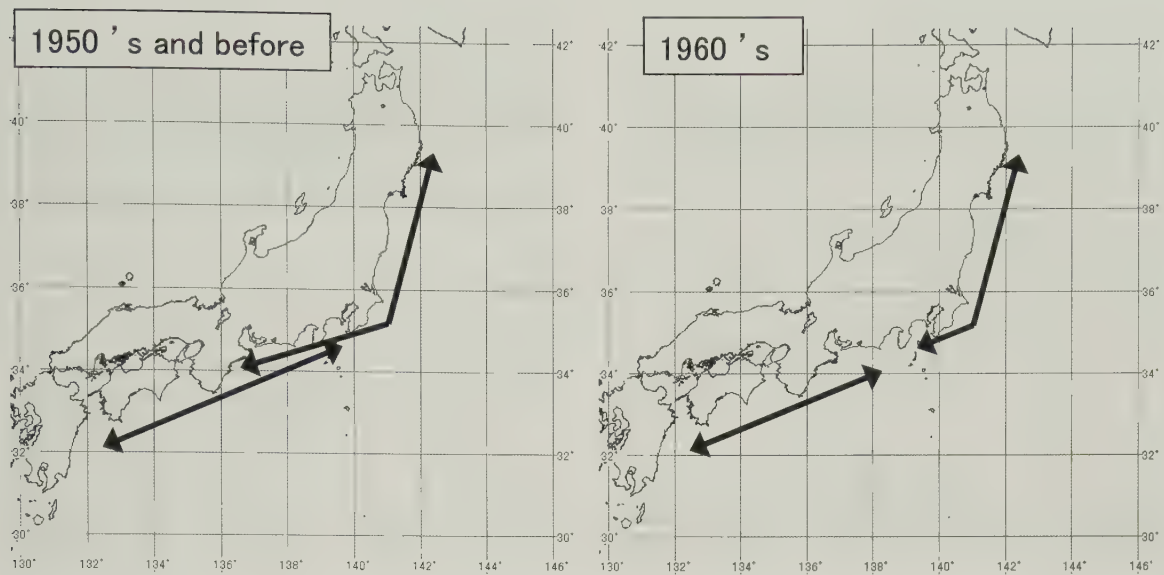


Fig. 11. A schematic diagram on the distribution and migration of over 3-year old yellowtail in the Pacific coast of Japan (after Tanaka, 1972a, b, and 1973).

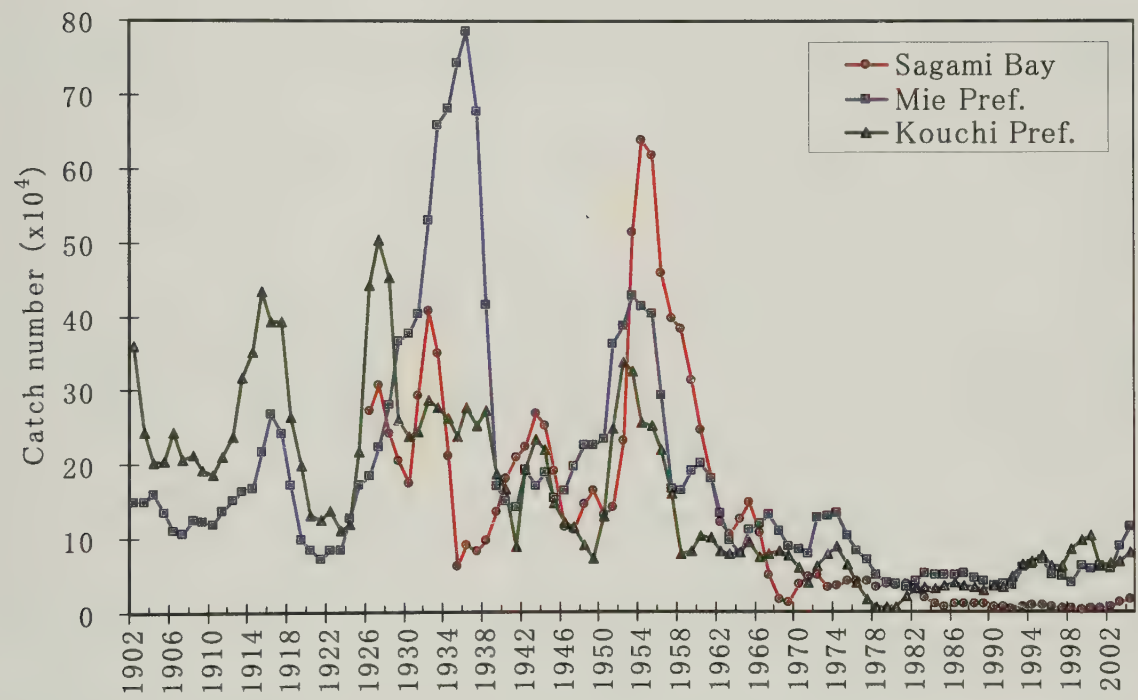


Fig. 12. Yearly catch number of Buri (yellowtail sized over 6kg) at main fixed netting areas along the Pacific coast of Japan (after Kuno, 2004 and original data). Figures are shown using three years moving average.

も、1960年代以前に比べて大きく減少するとともに、数十年の周期で豊凶がみられる（久野，2004）。太平洋側におけるブリ成魚の資源変動は、1956年を境にそれ以前の自然要因によると推定される変動と、それ以降の人為的要因によると推定される減少傾向とに分か

れるとされるが（木幡，1986），相模湾で1960年代以降に大型魚の漁獲量が三重県や高知県に比べて極端に少なくなったことと、大型魚の分布・回遊様式の変化が関連している可能性もあり、今後、海況変動と漁況の変化について検討する必要があるだろう。

第4章 今後の研究課題

これまで第2章、第3章においてブリの産卵場、初期生活史に関するレビュー、及び移動回遊に関するレビュー結果をみた。これらのレビューから整理された既往知見を Table 3~5に示す。

産卵生態に関して、レビューから得られた既往知見と未解明な点を Table 3に示す。産卵生態に関しては断片的な知見の集積から東シナ海、太平洋、日本海における産卵場の出現と移動のおおよその状況が推定されたが、データの蓄積は少なく海域別・時期別の産卵親魚の回遊時期・経路などは不明であり、適切な資源

Table 3. Summary of review on spawning biology of yellowtail

Item	Previous knowledge	Research subjects
Spawning period and area (information from adult fish)	Large size fish spawn earlier.	Does the suitable condition for spawning and spawning area differ by age?
	Spawning area moves northward as water temperature increases in the East China Sea.	
	They spawn along the edge of continental shelf in the East China Sea, at Danjyo Islands and Pacific Ocean (the Sea of Kumano). It is possible that they also spawn in the Sea of Japan (e.g. off Shimane Prefecture, Wakasa Bay and off Noto Peninsula).	Do all spawning adults belong the same population?
	The northern limits seem off Noto Peninsula and Izu Peninsula.	
Suitable water temp. for spawning	19~22°C (Danjyo Isls.-south of Goto Isls.).	What is the relationship between water temp. and maturation spawning activity and the survival rate?
	15~23°C (West of Kyushu, Tsushima Channel, south of Kagoshima Pref.).	
	19~21°C (after Mitani, 1960)	
Spawning period and area in the Sea of Japan (from eggs and larvae)	From the sea of Goto to Tsushima Channel: the peak period is in April and May.	When and where the young fish which occur in various places along the Sea of Japan are hatched?
	Southwest coast of Korea Peninsula: May (post-larvae)	
	Off Yamaguchi: May and after (pre- and post-larvae)	
	Off northern Kyushu: May and June (juvenile)	
	Around Oki Isls.: June and July (pre- and post-larvae)	
	The possibility that larvae spawned in the East China Sea in April and May have been transported to Tsushima Current side is high.	
	It may be distinguishable fish hatched in the Sea of Japan from fish hatched in the East China Sea by their hatching date. Fish are hatched in April and May in the East China Sea.	
Spawning period and area in the Pacific Ocean (from eggs and larvae)	South of Kagoshima Pref.: January-May	When and where the young fish which occur in various places along the Pacific Ocean are hatched?
	Main spawning area is the upper reaches of Kuroshio Current and southwestern area off Japan: pre- and post-larvae occur in April and May.	
	The Sea of Hyuga: pre- and post-larvae occur in April and May.	
	Northeastern area of Japan: pre- and post-larvae mainly occurred in June. Body length composition was intermittent (7-90mm).	How is the seasonal pattern of transportation, and how to estimate spawning area from hatching date in the Pacific Ocean area?
	Middle and southern part of the East China Sea: February and March. Off Kyushu and Shikoku: March-May. East of Shikoku: June.	
	The possibility that larvae spawned in the East China Sea in February and March have been transported to Pacific Ocean side is high.	

管理のためにはより詳細な調査・研究が必要である。産卵場の季節的な移動が引き起こされる原因については、冬～初夏にかけての水温変化であることは推測できるが、一方で適水温帯の全域が産卵場となるわけではなく、産卵場形成の必要条件については不明である。日本海側は春先から月が進むとともに産卵場が北上する様子が捉えられており、太平洋側についても明瞭ではないものの次第に西部から東部へ産卵場が移動ないし拡大する可能性がレビューの結果から示されており、産卵場に来遊する親魚の産卵回遊様式の解明については、手法の検討も含めて残された課題である。日本海側においても、太平洋側においても沿岸各地に来遊する主群が東シナ海生まれであるか、あるいは日本海、太平洋生まれであるかはっきりしたことはわかっていない。

流れ藻への随伴性を示すようになるモジャコ期の生

態については、随伴性発現、非発現のメカニズムなどが未解明の点である。0歳魚のブリがどの段階まで受動的に輸送されるのか、流れ藻に着いた個体と着かない個体で減耗率に差があるかなどが今後の課題と考えられる。

なお、今回のレビューでブリ属の卵、仔魚に関して外部形態による種査定の限界が既往知見の集約のネックになっていることが報告された。この問題は今後もフィールドでの標本を扱う際に問題となると考えられ、早期の査定技術開発が望まれる。

初期生活史については、食性、共食い、自発摂餌などに関する知見が得られている（Table 4）。来遊量予測技術開発や資源管理のためには、これらの成果を基礎にして更に加入量の変動機構解明に取り組む必要があるが、この分野の課題については既往成果も少なく今回は詳細なレビューの対象にならなかったが、今後

Table 4. Summary of review on early life ecology of yellowtail.

Item	Previous knowledge	Research subjects
Relationship with drifting seaweeds	A habit to follow drifting seaweeds occurs over 15mm.	
Two types, i.e. who follows and does not follow drifting seaweeds are observed.	What is the quantitative ratio of the two types?	
What is the difference of survival rate of the two types?		
Influence of juvenile catch	Juveniles (Mojoyako) are caught for aquaculture.	
Predation		
Growth	The difference of growth by school is observed (larvae produced in Tosa Bay is faster in growth).	Why does growth speed differ by regions?
Feeding habit	Small copepods etc. cannibalism occurs over 10mm.	What is the relationship between diet environment and survival rate?
Transportation	Stable from the East China Sea to the Pacific Ocean (February and March). Unstable to Tsushima Current side (May and June).	How is the process of distribution form the East China Sea to the two current?
How is the distribution rate decided?		
From where is recruitment in the sea of Japan and the Pacific Ocean supplied mainly?		
Physical environment		Is there relationship between the regime shift ?
Recruitment fluctuation factor		
Spawner-recruit relationship		

取り組むべき研究方向として重要であることは言を待たない。マイワシなど浮魚類ではレジームシフトと呼ばれる環境変化と、加入量変動、資源変動の関係が示唆されているが (Chavez *et al.*, 2003), ブリでも富山

湾において過去の漁況の長期変動と環境要因の長期変動の関係が示された。この現象は環境変動に伴うブリの回遊様式の変化による富山湾の漁況の変化なのか、加入量そのものの変動なのか、あるいは両方なのか、

Table 5. A summary of review on migration ecology of yellowtail

Item	Previous knowledge	Research subjects
The Sea of Japan: migration pattern by age	0-year old: spread as far as the coast of Hokkaido and the polar front. A range of movement after fish leave drifting seaweeds is small. Northern limit of wintering area is Sado Channel.	What is the suitable condition of distribution, the northern limit of migration, the condition of wintering area?
	1- and 2-year old: seasonal small scale migration, offshore and shore migration.	What is the suitable condition of distribution and the condition of wintering? Where is the northern limit of migration?
	3-year old: go south to spawning. The lower limit of water temperature in wintering area is 9°C.	What is the trigger to go to the south? Where is the southern limit of migration?
	4-year old and over: large scale migration from the East China Sea to Hokkaido is observed.	What is the relationship between migration and environment?
The Sea of Japan: fluctuation pattern of migration in decadal scale	1950s and 60s: 0-year old fish pass the winter in northern part of the Sea of Japan.	What is the relationship between long term fluctuation of environment?
	1970s and 1980s: 0-year old fish go south of Noto Peninsula in winter. They did not spread their distribution area to the north thereafter. The distribution area of adult fish reduced and young fish tend to distribute in west part of the Sea of Japan.	
	1990s: The catch of large sized fish increased in northern part of the Sea of Japan. Catch amount also increased in Toyama Bay. Water temp. was higher than the previous average.	
Pacific Ocean: migration pattern by age, by period	1960s and before: small sized fish (0- and 1-year old) seem to show two migration patterns i.e. that from Bousou Peninsula to Sanriku District and that of small scale movement in the west of Sagami Bay.	What was the migration pattern in 1970s and after?
	1950s and before: two patterns were observed fish over 3-years old, i.e. that from the sea of Kumano to Sanriku District and that from the Sea of Hyuga to Sagami Bay.	
	1960s: two patterns were observed fish over 3-years old, i.e. that from the sea of Sagami Bay to Sanriku District and that from the Sea of Hyuga to the Sea of Enshu. In Sagami Bay the catch amount of large sized fish decreased extremely.	Where are the spawning grounds?
	1970s and 80s: few data exist.	
	1990s: fish released in Wakayama Pref. went to Mie Pref. and Shikoku.	
	Catch fluctuation observed on a decadal scale.	Is the cause of catch fluctuation the change of migration pattern? What is the relationship between ocean environment and migration pattern?

今のところ明確ではなく解決すべき課題と考えられる。この課題への取り組みは過去データの収集と解析といった手法が可能である。

分布・回遊については過去の漁獲統計の解析、通常の標識放流調査、近年のアーカイバルタグを用いた標識放流の成果などから太平洋、日本海における未成魚、成魚についての知見が集積されつつあり、年齢によって分布域、回遊様式が異なること、同一年齢でも回遊様式は一通りではないこと、さらに長期間のデータを検討した場合、年代により変化が見られることなどが示されたがその詳細は課題として残されている (Table 5)。

年齢別の回遊については以下の問題点が指摘された。当歳のブリは稚幼魚期に流れ藻に付き比較的遠方まで輸送されるが、日本海側ではその後の1, 2歳時には移動範囲が狭く海域ごとに異なる回遊様式が見られるがそれは長期的に見て一定ではなく、回遊様式の詳細な把握と海洋環境との関係の解明が残された問題である。太平洋側でも相模湾以西と、北部では未成魚の回遊様式が異なる可能性もレビューされたが資料が少なく未解明の部分が多い。それぞれの海域の回遊様式の把握とその変動の解析、さらに回遊様式のあり方と海洋環境の関係の解明が課題として残されていると考えられる。

3歳に達すると日本海側については東シナ海への産卵回遊が開始されるが、太平洋側では三陸から遠州灘あるいは相模湾に分布する群と、日向灘から相模湾あるいは遠州灘に分布する群の2つがみられるのではないかと資料があり、親魚についても回遊様式の把握が必要である。さらに、産卵群として日本海から東シナ海へ回遊した群と太平洋の群の関係がこれまでの知見では必ずしもはっきりしていないという点が残された課題である。3章でみたように回遊様式に関して日本海でも太平洋側でも数十年規模の長期変動が報告されており、富山湾では、日本海西部における最低水温期の水温の長期変動と漁況の関連性が指摘されている。このように、海洋環境の変動状況とブリの回遊様式および漁況の変動状況からは、その関連性が示唆されるが、その他に、産卵場、加入量指数、モジャコ来遊量、日本海・太平洋への輸送（配分比率）などについてもこのような長期変動が存在するのか、過去データの解析から取り組むことは有益であろう。

このような年齢別の回遊様式の詳細を把握し、それと海洋環境との関係が解明されることで、資源評価調査の成果などから得られる年齢別資源量の情報と相まって、各地の漁業者に的確な来遊期、来遊量予測情報を提供することが可能となる。また、資源評価の基礎

となる個体群の構造についても理解が深まり再生産関係の把握などに役立ち、的確な資源管理につながることも期待できる。

第5章 ブリの産卵、回遊と海洋環境の関係を解明するための調査手法

第5章では、第2章と第3章にまとめられたこれまでの知見を踏まえ、第4章にあげられた研究課題を達成するための手法について述べる。個体の発生水域の推定や回遊様式の解明のためには、耳石微量元素分析やアーカイバルタグなど近年に開発された新たな手法が重要である。一方、年代による成熟や成長の変化を解明するためには、これまでに行われてきた古典的な手法の継続とその効率的な配置が重要である。

5-1 産卵場、産卵期の推定

2章に示したように、成魚の生殖腺の観察によって推定されている産卵期は、東シナ海の大陸棚縁辺から九州南岸では2～4月、九州西岸と四国から紀伊半島の太平洋沿岸では4～5月、山陰から能登半島周辺の日本海では6月以降である（三谷, 1960; 村山, 1992; 辻, 2000）。また、大型個体が東シナ海南部で早期に産卵し、中型個体が九州西岸でそれより遅く産卵することから（三谷, 1960）、産卵場の北上が産卵群の北上を意味するものではないと考えられる（上原ら, 1998）。しかし、これらの知見には50年近く前の結果も含まれていることや、確実に産卵を確認できる方法ではなかったことから、近年の海域別の産卵実態が詳細に把握されているとは言い難い。また、大型個体ほど高齢であると考えられることから、年齢によって産卵場が異なる可能性がある。つまり、産卵場の形成要因として産卵魚の年齢構成も重要となる。このため、村山（1992）、上原ら（1998）が行ったような大・中型まき網の漁獲統計の分析、さらには海洋環境資料との突き合わせによる検討も直ちに採りうる手法であるが、以下に示す親魚の年齢別成熟状況、卵仔稚の分布情報、海域別に来遊する当歳魚の誕生時期情報などを総合し、月別の産卵海域と産卵魚の年齢および水温を示したマップを作成することにより、近年における海域別の産卵実態を把握する必要がある。

成魚の生殖腺観察 ブリにおいて成熟状態から産卵場と産卵期を推定する場合、生殖腺の熟度階級の判定、および熟度階級と生殖腺熟度指数の対応が用いられてきた。しかし、産卵の有無を正確に判断するためには、組織学的観察により排卵後ろ胞を確認すべきである。また、ブリでは未だ成熟状態の組織学的規準

が確立されていないため、生殖周期や天然海域での一産卵期当たりの産卵数を見積もることができない。今後は、飼育魚を用いて成熟状態の組織学的規準を明らかにする必要がある。また、天然海域での産卵状況調査では、この規準を用いてより正確な情報の提示が望まれる。特に、産卵場の北縁部である若狭湾から能登半島周辺および紀伊半島から伊豆周辺での情報が重要である。また、東シナ海南部での産卵終期の確認も必要である。海域別の調査実施期間は、東シナ海中部では2～5月、九州西岸では3～6月、日本海西部では4～7月、日本海中部では5～7月、薩南-日向灘では3～5月、四国周辺・熊野灘・伊豆周辺では3～5月が適当であろう。但し、近年では我が国の漁業の変化により東シナ海南部における標本入手が難しくなっている。

産卵魚の年齢査定には、耳石、鱗、鰓蓋骨、脊椎骨に形成される輪紋を用いる方法があるが、輪紋の観察の容易さや体長との関係のばらつきの小ささなどから、鰓蓋骨と脊椎骨が適している（三谷, 1960）。脊椎骨の場合、体長に対する成長率が最も大きいことから第15～18椎体が適している（三谷, 1960）。これまでの研究例では第17椎体を用いているので（三谷, 1960；村山, 1992）、比較のために今後も第17椎体を使用することが望ましい。ただし、ブリでは個体の成長が5歳以上で頭打ちとなるので、上述のどの年齢形質を用いても5歳以上の年齢分解は困難である（三谷, 1960）。したがって、詳細な年齢査定のために新たな年齢形質を検討する必要がある。甲殻類（シャコ）では経時的に脳内に蓄積されるリポフシン量を測定することにより、年齢査定が可能となっている（Kodama, *et al.* 2005）。魚類でもこのような個体の成長に因らない年齢査定法の確立が望まれる。

調査船による産卵調査および卵稚仔の同定技術の向上 月別・発生段階別の卵と仔魚の分布マップの作成により、産卵場からの卵・稚仔の分散状況を知ることができる。

水産研究所と都道府県水産試験場では、長年にわたって調査船による魚類の卵・稚仔採集調査が行われている。この調査は主に多獲性魚類であるイワシ類やサバ類の卵と稚仔を対象としたものであり、LNP（改良型ノルパックネット）の鉛直曳きによる採集が多くの定点で行われてきた。この調査ではブリの卵や仔魚の採集位置や採集数は記録されてこなかったが、実際にはブリも含まれていた可能性がある。LNPによる標本が利用できれば、卵と仔魚の分布状況を把握することが可能となる。ブリ属の卵仔稚の分布水温は19～22℃台に集中していることから、卵仔魚採集調査ではそ

の水温を示す調査水域と調査時期を中心に行うことが必要である。

ブリの卵仔稚魚の採集海域では、その他のブリ属であるヒラマサやカンパチの仔稚魚が採集される（東海区水産研究所, 1967；東海区水産研究所・南西海区水産研究所, 1970；浅見ら, 1967）。しかし、ブリの卵は未だ種の同定が可能となっておらず、形態学的な詳しい比較による同定の可能性を見極める必要がある。仔魚期以降では、カンパチとの判別は容易であるが、体長13mm程度まではヒラマサと酷似しており、それらの判別には注意が必要である。卵・仔魚とも形態による種判別が不可能な場合は、モノクローナル抗体による判別手法の開発やDNA等遺伝情報による判別を検討する必要がある。モノクローナル抗体を用いた種判別はアサリなど二枚貝で開発されているが（浜口, 1999）、浮遊性魚卵の同定も可能であることは大西ら（2003）によって示されている。

耳石の日周輪解析による産卵場の推定 Pannella（1971）により魚類耳石の日輪構造が示唆されたが、ブリの耳石でも1日1本の日周輪が形成されることがわかっている（Sakakura and Tsukamoto, 1996）。これを解析することにより仔稚魚の日齢や成長履歴を推定することができる。

産卵場で生まれたブリは、流れ藻に付くモジャコとなって分散する。モジャコ採捕漁業の主漁期は、九州西岸と三重県以西の太平洋側では4～5月、日本海側と静岡県以东の太平洋側では6～7月である。耳石日周輪の解析によると、太平洋側で漁獲されるモジャコは2～3月に、日本海側で漁獲されるモジャコは4～5月に生まれたものが中心となっている（村山, 1992）。前述のように、産卵期は太平洋側で5月まで、日本海側で6～7月まで続くと考えられることから、モジャコ採捕漁業によって採捕されるものよりも遅く生まれた群も存在すると考えられる。モジャコ期を過ぎた幼魚は、初夏以降に沿岸の定置網やまき網で漁獲され始め、これらが成長と移動を行いながら各地で漁獲される、すなわち加入主群となると推察される。したがって、耳石日周輪の計数によってこれらの幼魚の誕生日組成を把握すれば、産卵生態の解明によって得られた月別の産卵海域マップから、それらの生まれた海域の範囲を推定することができる。日本海における産卵期は東シナ海と比べてかなり遅いことから、この手法は日本海で漁獲される幼魚において特に有効であろう。

ブリ未成魚の成長は水温の影響を受け、暖かいほど早く成長する（村山, 1992）。水温と初期成長の関係が耳石中心部の日周輪間隔に反映されていれば、これ

を解析することにより発生した水域の水溫環境を推定することができる可能性がある。このためには、人工種苗の水溫を違えた飼育実験により、耳石の輪紋間隔として残される水溫による初期成長の違いを把握する必要がある。知られている産卵場の水溫から、13～26℃の範囲にいくつかの実験区を設定するのが適当であろう。各実験区の設定水溫下において受精卵を得る技術の開発がこの実験の鍵となろう。この結果と天然魚の耳石中央部の輪紋間隔測定によって得られた初期成長を比較し、天然魚の生まれた当時の水溫環境を推定することにより、成魚の成熟状態や卵稚仔の分布によって推定される比較的広い産卵水域から、加入主群が発生した水域を絞り込むことができると考えられる。

ブリ耳石の日周輪は比較的観察しやすく、体長60mm程度までなら研磨を行う必要はない。それ以上の体長のものは縁辺部を傷つけないようにsagittal面の研磨を行う。輪紋数が多いであろうと思われる比較的大きな個体においてはtransverse面の研磨が適しているが、研磨面の選択基準となる体サイズは決定されていない。輪紋数の計数や輪紋間隔の測定には、Latok社等の耳石解析システムを用いる。

耳石微量元素組成の解析 耳石は、魚の齢や成長履歴だけでなくその経験した環境を記録しており、微量元素組成の分析により耳石に記録された環境を推定することができる (Campana, 1999)。例えば、カタチイワシではストロンチウム－カルシウム比の分析により発生海域の塩分が推定できる (Kimura *et al.*, 2000)。ブリにおいても、生まれた頃に形成される耳石の核付近の微量元素組成を把握することにより、産卵海域を推定することができる可能性がある。このためには、産卵海域ごとにそこで生まれたことが明らかな標本を採集し、それぞれの耳石微量元素組成を明らかにしておく必要がある。また、太平洋側と日本海側では水溫などの環境が異なるので、発生から漁獲されるまでの回遊様式を推定できる可能性がある。この場合もそれぞれの水域で漁獲された標本の微量元素組成を明らかにしておく必要がある。

耳石微量元素分析には2つの方法がある。すなわち、EPMA (Electron Probe Micro-Analysis) では微量部分における分析が可能で、耳石上に微量元素のマッピングを行うことができる。このため、耳石上における任意の位置の情報を示すことができ、環境と魚の成長履歴との対応を見ることができる。ICP-MS (Inductively Coupled Plasma Mass Spectrometry) はEPMAより分析精度に優れているが、試料全体の微量元素組成を示す。このため、中心部分など耳石から必要部分を摘出する技術の開発が必要である。

ポップアップアーカイバルタグを用いた産卵場推定

ポップアップアーカイバルタグは、曳航索を伴った鉤先とともにタグを魚体に射ち込み、魚にタグを曳航させる方法で装着し、一定期間を経過した後に曳航索が切り離されてタグが水面上に浮上し、人工衛星を介して期間中に得られたデータと浮上位置を送信するタグである。標識個体が再捕されなくてもデータが得られる点で有用であり、マグロ類、カジキ類、サメ類の標識放流調査に用いられている (高橋、斉藤, 2003)。また浮上した地点についてはアルゴシステムにより正確に把握することが可能である。現状ではタグ本体部の長さが約17cm、アンテナ長が17cmあるために、成魚でも魚体重が10kg台であるブリへの装着は魚体への負担が大きいと考えられていた。しかし、ダミータグを装着したブリ成魚の飼育実験では、タグの装着が魚体に与える負担はそれほど大きくないという結果が得られつつある (阪地ほか, 未発表)。このタグを産卵のために南下中の成魚に装着し、産卵期間中に曳航索が切り放されるようにセットできれば、産卵回遊と産卵場の関係解明が進むものと思われる。

5-2 卵稚仔の輸送

これについては調査船による現場での卵稚仔採集調査を挙げられる。一定の定点で定期的に採集を行う方法と、特定の水塊に含まれる卵稚仔を追跡する方法がある。これらの手法は今後も調査・研究の中心であることは言を待たないが、以下にブリに適用可能な別の手法を挙げる。

一つはデータ配信型の漂流ブイを産卵場付近に投入してその挙動を観察することが、卵稚仔の輸送過程を解明するために重要な手法である。産卵場に到達する前の親魚にポップアップタグ (前出) を装着し、産卵期間中に切り放されたものをそのまま追跡するという手法も考えられる。切り放されたタグは、電池の寿命に依存するが、浮上したまま2週間から1月程度は電波を発信可能であると思われる。

マアジでは、東シナ海から太平洋並びに対馬暖流域への卵稚仔の輸送過程をコンピュータ上で再現する試みが行われている (Komatsu and Kasai, 2005)。産卵海域での海水流動モデルが開発されれば、ブリでもシミュレーション実験が可能である。

また、間接的な方法ではあるが、沿岸各地に來遊した主群の日齢査定から誕生月を把握し、5-1で述べた月別産卵場マップと照合し輸送経路を推定することも可能かもしれない。

モジャコ期の生態 これまでにモジャコ及び流れ藻への標識放流調査が行われ、流れ藻に随伴するモジャ

コの移動が明らかにされた(農林水産技術会議事務局, 1967)。また, 航空機を用いた流れ藻量の観測によるモジャコ資源量の推定が試みられた(三谷, 1965a, b, c, 1968)。しかし, 第2章に示したとおり, 必ずしも全ての個体が流れ藻に随伴するわけではない。どの程度の個体が流れ藻には着かず, それらの個体はどのような移動を行うのかについては, 表層あるいは中層トロール網など, ある程度以上の速度を有し, 濾水量の大きな採集器具による調査が必要であろう。流れ藻への随伴性発現機構については, 照度を変えた飼育実験などの手法も考えられる。

5-3 移動・回遊の解明

漁獲統計資料および魚体測定資料の解析 ブリでは, わかし, いなだ, わらさ, ぶりなどほぼ年齢に対応する銘柄別統計が存在する地域がある。また, 主に都道府県水産試験場による体長組成データも蓄積されている。これら銘柄別漁獲量や体長組成データは地域によるブリの相対的重要性の違いによる偏在性が著しいものの, 成長段階別の分布の把握によって回遊様式の概要を推定することができる。第3章に述べた対馬暖流域における回遊様式の年代による変化は, 主にこの手法と次に述べる外部標識を用いた放流調査の結果を用いて明らかにされた。この手法は早急に開始できる点で有効であるため, 新たな資料の掘り起こしを行い, 東シナ海や太平洋における回遊様式の変化も検討するべきであろう。

外部標識を用いた放流調査 古くから, ブリは大規模な回遊を行うものとされ, 回遊の経路や時期について漁業者の関心および研究ニーズが大きく, 太平洋では1917年, 日本海においては1918年から金属製および樹脂製の外部標識を用いた標識放流調査が行われてき

た。田中(1973)は, 太平洋側の各地において1926年から1938年に行われた1,024個体の標識放流調査結果を取りまとめたほか, 田中(1972a)は, 宮崎県から岩手県の範囲において, 1963年から1965年の3年間にわたって1,422個体の標識放流調査を行った。渡辺(1979)は, 1923年から1971年にかけて対馬暖流域の各地において行われた4,592個体の標識放流調査結果を取りまとめた。現在考えられているブリの回遊様式は, これらの標識放流調査の結果と漁況や漁獲物の季節変動から推定されたものである。樹脂や金属製の外部標識による調査は, 放流から再捕にいたるまでの標識魚の行動を把握することができないため, 短期的な標識魚の移動を把握するには有効であるが, これまでに知られている以上に詳細な移動・回遊に関する知見を得ることは難しいと考えられる。

アーカイバルタグを用いた放流調査 近年, 自らの位置推定を行い, そのデータを記録することが可能な, アーカイバルタグと呼ばれる集積回路内蔵の記録型標識が開発された。これにより, 放流から再捕までの標識魚の遊泳位置, 遊泳水深および水温を連続的に把握することが可能となった。2005年現在, 得られたデータの信用性が確認されるとともに, クロマグロおよびブリの標識放流調査に用いられるなど, 我が国における使用実績を有するアーカイバルタグ(Fig. 13)は, カナダのLOTEK社製のみであり, 現行モデルのLTD2310タイプは, 本体部の長さ76mm, 直径16mm, センサーケーブルの長さ220mm, 重量45g, メモリ容量8MB, 電池寿命6.5年である。これまで, クロマグロでは尾叉長45g以上の個体に, ブリでは尾叉長52cm以上(井野ら, 未発表)の個体に装着された実績があり, その成果がInagake *et al.* (2001), Itoh *et al.* (2003a), Itoh *et al.* (2003b)によって報告

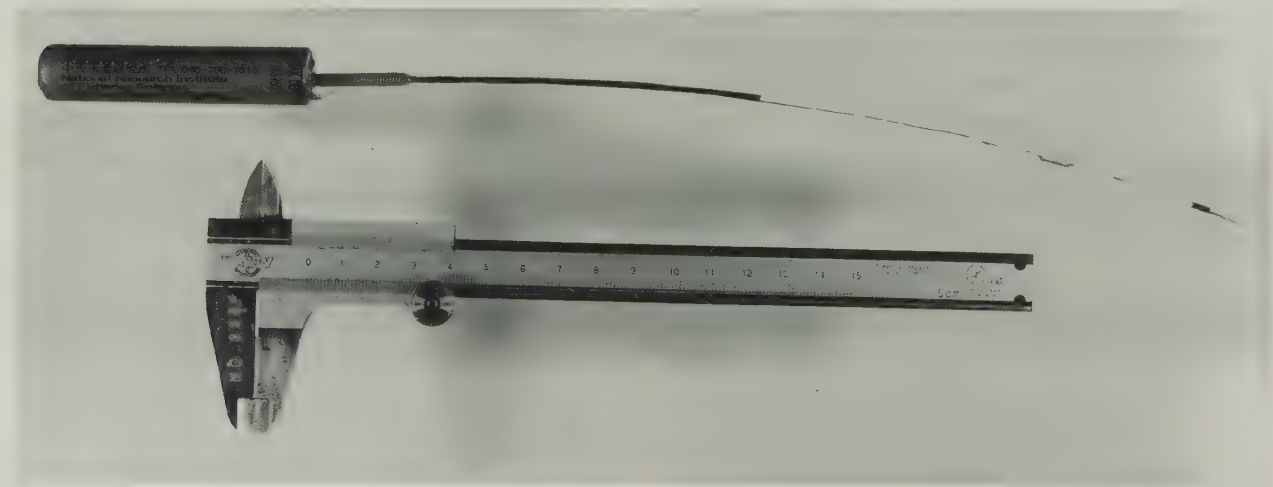


Fig. 13. Photograph of an archival tag (LTD2310).

されている。このタグによる経度の推定値は約1°の信頼性を有するが、緯度の推定値の誤差は経度誤差と比較して大きく、クロマグロにこのタグを用いた場合においては、タグから得られた経度値と水温値を表面水温の分布図と照合して緯度を推定することによって、およそ1°の誤差で緯度が推定できる (Itoh *et al.*, 2003a)。Inagake *et al.* (2001), Itoh *et al.* (2003a), Itoh *et al.* (2003b) は、このタグを用いた調査結果から、日本列島周辺におけるクロマグロ若齢魚の移動状況を示し、対馬海峡で放流した標識魚が東シナ海で冬季を過ごした後日本海を北上し、北海道西岸沖へ移動した事例等を報告しているほか、移動や遊泳行動と水温すなわち海洋環境の関連性について検討しており、その有用性が示されている。ブリの移動・回遊状況を明らかにするための調査においても、このタグは有用であると考えられる。このほか LOTEK 社からは、前述のタイプに加え、本体部の長さ37mm、直径11mm、センサーケーブルの長さ100mm、重量10g、メモリ容量384KB、電池寿命2年の小型機種 (LTD2410) が2002年から製造販売されている。この機種は、これまでよりも小型の個体への装着が可能であると考えられ、キハダへの装着実験では、尾叉長30cm程度の個体に装着できる可能性が示されており (新田, 2003)、その結果からみて、ブリについては、0歳魚 (尾叉長30~40cm) への装着が可能であろう。

5-4 海洋環境調査

ブリの産卵場・発生水域・位置等を解明するためには海洋環境との関係进行分析が必要があり、分布域全体にわたる海洋環境を明らかにする必要がある。特に、回遊様式の把握においては、前述のアーカイバルタグによる回遊様式の推定精度を向上させるために、豊富な水深別の水温データが必要である。さらに、産卵場の形成要因や太平洋と日本海への仔稚魚の分配機構を明らかにし、環境による産卵場や加入量の変化を予測するためには、長期にわたる海洋環境調査が必要不可欠である。現在、水産総合研究センターをはじめ多くの研究機関が海洋観測のための定線調査などを継続して行っており、このデータを利用することが出来る。

また、各地の定置網漁場では漁況予測のために定置水温の時系列データが採取されていることが多い。ブリの回遊や行動を明らかにするためには、各地で想定されている水温と漁況の関係を一般化することも重要である。

人工衛星情報 (リモートセンシング) 人工衛星を利用した表面水温、海色、海面高度などの調査が可能であるが、ブリの産卵・回遊と環境の関係を研究する場合には表面水温の面的把握が重要である。表面水温に関して現在最も一般的に利用できるデータには米国の人工衛星である NOAA から受信したものがある。この画像は、日本においても幾つかの機関が受信し、適宜処理を行った後有料あるいは無料で公開してい

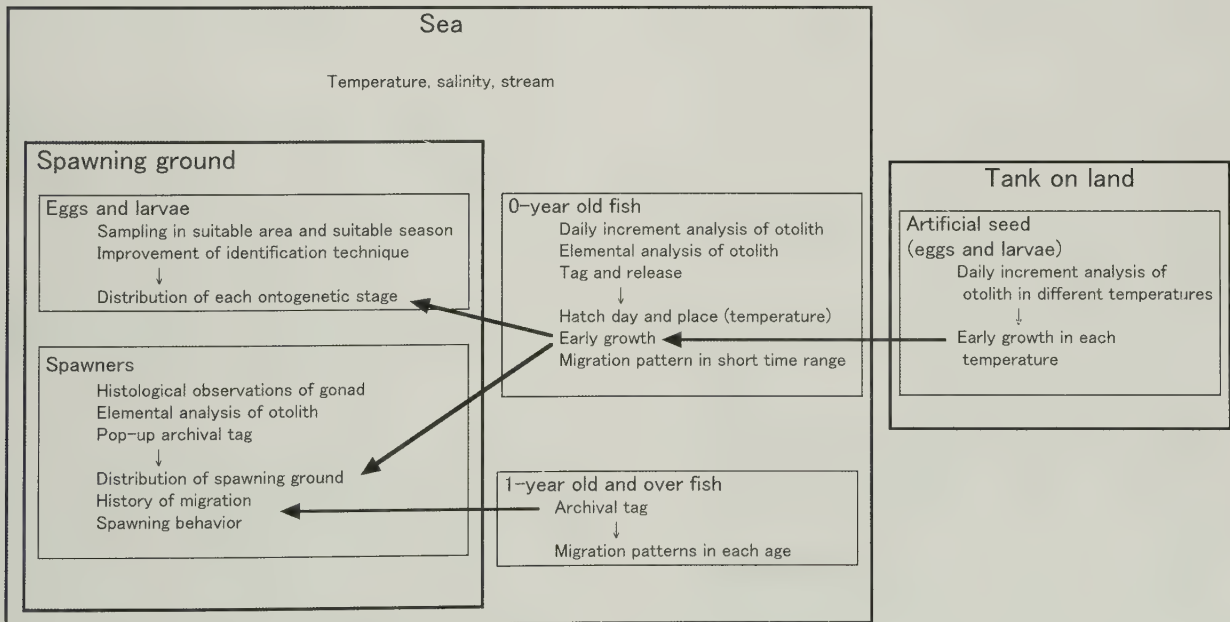


Fig. 14. Research methods for the definition of the relationship between migration and spawning pattern of yellowtail *Seriola quinqueradiata* and environmental features.

る。本州周辺の画像については水産総合研究センター日本海区水産研究所のホームページ^{*4}からもダウンロードが可能である。このデータを地理情報システムと組み合わせることで(西田ら, 2004)ブリの回遊と環境に関する研究の発展が図れるものと考えられる。

以上に述べた手法とその対象となるブリの成長段階を示す (Fig. 14)。

ブリの産卵, 回遊生態を解明するためには, 産卵場・産卵時期の解明, 産卵場からの卵稚仔の輸送の解明, 各地の来遊主群の由来の解明, 沿岸各地に漂着して以降の移動・回遊の解明が必要であり, それらの課題・手法について考察を行ったが, 移動・回遊については成長段階別に把握する必要がある, また海洋環境との関係の解析も不可欠であることが示された。以上のことが明らかになれば漁況予報など実用面への波及効果は大きいと思われる。漁況予測手法, 資源管理手法を高度化するためには数量的な変動の把握とその要因解明についても明らかにしなくてはならないが, この部分は第2章のレビューでもわかるとおり未着手法部分が多い。Fig. 14にも示したとおり初期生態の解明には飼育実験を用いる手法も考えられるが, ブリでは親魚飼育, 自然採卵の技術が確立されており, 飼育実験が可能な魚種と言える。

ブリは幼魚から産卵親魚に至る様々な成長段階で漁獲されており, 調査の目的や手法も成長段階毎に異なる。ブリの資源変動と海洋環境の関係を明らかにするためには, どの成長段階への調査も必要である。水産総合研究センターが中心となって, 多くの研究機関や漁業関係者の協力体制を構築することが望まれる。

文 献

- 安楽正照, 畔田正格, 1965: 流れ藻に付随するブリ稚仔魚の食性, 西海水研報, 33, 13-45.
- 浅見忠彦, 花岡藤雄, 松田星二, 1967: モジャコ採捕のブリ資源に及ぼす影響に関する研究-(特) 産卵および発生初期の生態ならびにモジャコ漁獲および減耗に関する研究-1) 産卵および発生初期の生態ならびにモジャコの標識放流に関する研究. 研究成果30, 農林水産技術会議事務局, 1-61.
- Campana S.E., 1999: Chemistry and composition of fish otoliths: pathways, mechanisms and applications. *Mar. Ecol. Prog. Ser.*, **188**, 263-297.
- Chavez F.P., Ryan J., Lluch-Cota S.E., Niguen M., 2003: From anchovies to sardines and back: multidecadal change in the Pacific Ocean. *Science*, **299**, 217-221.
- Cho S.H., Myoung J.G., Kim J.M., and Lee J.H., 2001: Fish fauna associated with drifting seaweed in the coastal area of Tongyeong, Korea. *Trans. Am. Fish. Soc.*, **130**, 1190-1202.
- 藤田矢郎, 森 勇, 1982: 天然ブリ仔資源保護培養の為の基礎調査実験. 昭和56年度報告, 日裁協研究資料, 53-80.
- 深滝 弘, 1958: 対馬暖流水域におけるブリ稚魚の出現・分布について. ていち, **16**, 35-45.
- 花岡藤雄, 1995: ブリの産卵とモジャコの来遊機構. 宮崎県水試研報, **146**, 1-44.
- 原田輝雄, 1965: ブリの増殖に関する研究. 近大農紀要, **3**, 1-291.
- 原 哲之, 1990a: 日本海へ来遊するブリ成魚の来遊量指数とその年変動. 日水誌, **56**, 19-24.
- 原 哲之, 1990b: 日本海におけるブリ若齢魚漁獲量の年変動. 日水誌, **56**, 1933-1939.
- 原 哲之, 1990c: 日本海沿岸域におけるブリ成魚漁獲量の年変動について. 日水誌, **56**, 25-30.
- 原 哲之, 村山達朗, 1992: 日本近海におけるブリ来遊量の長期変動. 日水誌, **58**, 2219-2227.
- 服部茂昌, 1964: 黒潮ならびに隣接海域における稚魚の研究. 東海区水研報, **40**, 1-158. 浜口昌巳, 1999: 瀬戸内海アサリ漁場生態調査における適用方法の開発. 魚介類の初期生態解明のための種判別技術の開発. 農林水産省農林水産技術会議事務局, 東京, 66-76.
- 一丸俊雄, 田代征秋, 永谷 浩, 山本憲一, 1993: 対馬周辺におけるブリ1歳魚の移動回遊と飼付漁業の漁獲量変動. 西海ブロック魚海況調査研究報告, **2**, 11-23.
- 池原宏二, 1977: 佐渡海峡水域の流れ藻に付随する魚卵, 稚魚. 日水研報, **28**, 17-28.
- 今井貞彦, 1954: 鹿児島大学水産学部対馬暖流開発調査資料, **1**, 67-71.
- 今井貞彦, 1955: 鹿児島大学水産学部対馬暖流開発調査資料, **2**, 52-75.
- Inagake D., Yamada H., Segawa K., Okazaki M., Nitta A., Itoh T., 2001: Migration of young bluefin tuna, *Thunnus orientalis* Temminck et Schlegel, through archival tagging experiments and its relation with oceanographic conditions in the

^{*4} <http://www.jsnf.affrc.go.jp/cgi-bin/noaajpg.cgi?jpg+I2006+> 日本周辺画像

- western north Pacific. *Bull. Nat. Res. Inst. Far Seas Fish.*, **38**: 53-81.
- 井野慎吾, 2003: ブリ資源の管理に向けて－アーカイバルタグを使用した回遊生態調査－. 農林水産研究ジャーナル, **26**, 40-43.
- 井野慎吾, 河野展久, 奥野充一, 2006: 2. 海洋環境と回遊, 「水産学シリーズ 148, ブリの資源培養と養殖業の展望」, 恒星社厚生閣, 東京, 22-31.
- 石田 実, 武田保幸, 井元栄治, 平田益良雄, 田中七穂, 森由基彦, 黒木敏行, 野島通忠, 上原伸二, 1997: 1978年から1995年までの南日本太平洋沿岸の浮魚類卵仔稚の分布. 南西海区水産研究所, 206pp.
- Itoh T., Tsuji S., Nitta A., 2003a: Migration patterns of young Pacific bluefin tuna (*Thunnus orientalis*) determined with archival tags. *Fish. Bull.*, **101**: 514-534.
- Itoh T., Tsuji S., Nitta A., 2003b: Swimming depth, ambient water temperature preference, and feeding frequency of young Pacific bluefin tuna (*Thunnus orientalis*) determined with archival tags. *Fish. Bull.*, **101**: 535-544.
- 伊東祐方, 1959: 丹後伊根浦の冬ブリ漁況の長期変動について. 日水研年報, **5**, 29-37.
- 加藤義雄, 1954: 京都府沖合の稚魚の出現時期並びに出現傾向について. 対馬暖流開発調査第1回シンポジウム発表論文集, 247-250.
- 加藤義雄, 1955: 京都府沖合の魚卵・稚魚について. 対馬暖流開発調査第2回シンポジウム発表論文集, 105-108.
- 加藤史彦, 渡辺和春, 1985: 日本海におけるブリ資源の利用実態とその改善. 漁業資源研究会報, **24**, 99-117.
- 川村久明, 1955: 東対馬水道に於ける稚魚の季節的出現傾向. 対馬暖流開発調査第2回シンポジウム発表論文集, 83-94.
- 木村喜之助, 1952: ブリの産卵場・産卵期に関するブリ卵巢の調査. 東北水研報, **1**, 54-62.
- Kimura, R., Secor, D.H., Houde, E.D., Piccoli, P.M., 2000: Up-estuary dispersal of young-of-the-year bay anchovy *Anchoa mitchilli* in the Chesapeake Bay: inferences from microprobe analysis of strontium in otoliths. *Mar. Ecol. Prog. Ser.*, **208**, 217-227.
- 北原 武, 原 哲之, 1990: 回遊性資源の来遊量指数. 日水誌, **56**, 1927-1931.
- 木幡 孜, 1986: ブリ太平洋系群成魚の長期減少傾向について. 日水誌, **52**(7), 1181-1187.
- 木幡 孜, 1987: 太平洋主要3海域の大型定置網によるブリ成魚漁獲量の海域間相関. 日水誌, **53**(2), 215-218.
- 児玉純一, 2003: 沿岸の環境と生態系に関するモニタリング－(2) 宮城県における海洋環境モニタリングの重要性－. 水産海洋研究, **67**(3), 190-193.
- Kodama K., Yamakawa T., Shimizu T. and Aoki I., 2005: Age estimation of the wild population of Japanese mantis shrimp *Oratosquilla oratoria* (Crustacea: Stomatopoda) in Tokyo Bay, Japan, using lopofuscin as an age marker. *Fish. Sci.*, **71**, 141-150.
- Kohbara J., Hidaka I., Matsuoka F., Osada T., Furukawa K., Yamashita M., and Tabeta M., 2003: Self-feeding behaviour of yellowtail, *Seriola quinqueradiata*, in net cages: diel and seasonal patterns and influences of environmental factors. *Aquaculture*, **220**, 581-594.
- Komatus K., Kasai A. and Watanabe T., 2005: Modeling transport of eggs and larvae of jack mackerel in the East China Sea. North Pacific Marine Science Organization 14th annual meeting, Vladivostok, RUS, Program and abstract, 54.
- 小西芳信, 2000: 流れ藻は中国からもやってくる. 西海水研ニュース, **103**, 11-15.
- 久野正博, 2004: ブリ資源の長期漁獲変動とレジームシフト. 黒潮の資源海洋研究, **5**, 29-37.
- 松田星二, 1969: 南西海区水域に出現する魚卵・稚魚の研究－I. 南西海区水研報, **2**, 49-83.
- 三谷文夫, 1960: ブリの漁業生物学的研究. 近畿大学農学部紀要, **1**, 81-300.
- 三谷文夫, 1965a: 航空観察によって得られた流れ藻量からモジャコ資源量を推定する1つの試み－I. 日水誌, **31**, 423-428.
- 三谷文夫, 1965b: 航空観察によって得られた流れ藻量からモジャコ資源量を推定する1つの試み－II. 日水誌, **31**, 429-434.
- 三谷文夫, 1965c: 航空観察によって得られた流れ藻量からモジャコ資源量を推定する1つの試み－III. 日水誌, **31**, 500-505.
- 三谷文夫, 1968: 航空観察によって得られた流れ藻量からモジャコ資源量を推定する1つの試み－IV. 日水誌, **34**, 324-334.
- 村山達郎, 1992: 日本海におけるブリの資源生態に関する研究. 島根県水試研報, **7**, 1-64.

- 村山達朗, 北原 武, 1992: プリ来遊量の長期傾向. 日水誌, **58**, 409-416.
- 永田俊一, 1959: 日本海におけるブリ標識放流結果について. 日水研報, **7**, 43-55.
- 日本栽培漁業協会, 1999: プリ種苗放流技術開発調査. 日栽協研究資料, **75**, 日栽協, 138pp
- 西田 勤, Meaden G., 伊藤喜代志, 2004: 海洋 GIS と空間解析－水産海洋分野における現状と展望－. 月刊海洋, **407**, 340-345.
- 新田 朗, 2003: 新型アーカイバルタグ装着試験. 平成14年度日本周辺高度回遊性魚類資源調査委託事業報告書, 独立行政法人水産総合研究センター, 251-254.
- 農林水産技術会議事務局, 1967: モジャコ採捕のブリ資源に及ぼす影響に関する研究. 研究成果, **30**, 1-148.
- 能津純治, 工藤勝宏, 林 泰行, 篠田 保, 伊藤公雄, 小中邦夫, 1967: モジャコ採捕のブリ資源に及ぼす影響に関する研究－Ⅰ 産卵および発生初期の生態ならびにモジャコ漁獲および減耗に関する研究－2) モジャコおよび流れ藻の分布と海況. 研究成果, **30**, 農林水産技術会議事務局, 62-69.
- 小達 繁, 1962: 東北海区における稚魚の研究Ⅱ. アジ科 Carangidae. 東北水研報, **20**, 94-105.
- 小達 繁, 1967: 東北海区における稚魚の研究Ⅳ. 出現種類と季節的出現傾向. 東北水研報, **27**, 61-75.
- 沖山宗雄, 1965: 佐渡海峡に出現する魚卵・稚仔に関する予察的研究. 日水研報, **15**, 13-37.
- 大西庸介, 池田知司, 広石伸互, 沖山宗雄, 2003: モノクローナル抗体を用いた浮遊性魚卵の同定. Nippon Suisan Gakkaishi, **69**, 170-177.
- Pannella G., 1971: Fish otolith: daily growth layers and periodical patterns. *Science*, **173**, 1124-1127.
- Safran P., 1990: Drifting seaweed and associated ichthyofauna: floating nursery in the Tohoku waters. *La Mer*, **28**, 225-239.
- Safran P. and Omori M., 1990: Some ecological observations on fishes associated with drifting seaweed off Tohoku coast, Japan. *Mar. Biol.*, **105**, 395-402.
- Sakakura Y. and Tsukamoto K., 1996: Onset and development of cannibalistic behaviour in early life stages of yellowtail. *J. Fish Biol.*, **48**, 16-29.
- 阪地英男, 山本敏博, 2006: Ⅱ. 漁業資源-5. 資源評価と管理. 「水産学シリーズ 148, ブリの資源培養と養殖業の展望」, 恒星社厚生閣, 東京, 53-63.
- Sakakura Y. and Tsukamoto K., 1997: Age composition in the school of juvenile yellowtail *Seriola quinqueradiata* associated with drifting seaweeds in the East China Sea. *Fish. Sci.*, **63**, 37-41.
- 沢田郁次, 石津 峻, 田中 暲, 吉川明夫, 1960: プリ資源調査報告(1). ていち, **26・27**, 101-153.
- 西海区水産研究所, 1966: 九州西海域・東シナ海域における主要魚種の卵稚仔魚分布－Ⅱ (1965年2～7月採集). 西海区水産研究所資料集, 75pp.
- 西海区水産研究所, 1967: 九州西海域・東シナ海域における主要魚種の卵稚仔魚分布－Ⅲ (1966年1～7月採集). 西海区水産研究所資料集, 62pp.
- 西海区水産研究所, 1969: 九州西海域・東シナ海域における主要魚種の卵稚仔魚分布－Ⅳ (1967年1～7月採集). 西海区水産研究所資料集, 57pp.
- 千田哲資, 1962a: 隠岐等近海に於ける魚卵・稚魚の出現について－Ⅰ 出現する種類. 日本生態学会誌, **12**, 152-157.
- 千田哲資, 1962b: 隠岐等近海に於ける魚卵・稚魚の出現について－Ⅱ 季節変化. 日本生態学会誌, **12**, 163-166.
- 千田哲資, 1965: 流れ藻の水産的利用. 水産研究叢書, **13**, 日本水産資源保護協会, 55pp.
- Shimomura T. and Fukataki H., 1957: On the year round occurrence and ecology of eggs and larvae of the principal fishes in the Japan Sea-1. *Bull. Japan Sea Reg. Fish. Res. Lab.*, **6**, 155-290.
- 高橋末緒, 斉藤宏和, 2003: ポップアップ式衛星通信型タグによるまぐろ・かじき類調査の現況. 遠水研ニュース, **112**, 18-23.
- 田中昌一, 1967: モジャコ採捕のブリ資源に及ぼす影響に関する研究 - (特) 産卵および発生初期の生態ならびにモジャコ漁獲および減耗に関する研究－3) モジャコ漁場における流れ藻の標識放流. 研究成果, **30**, 農林水産技術会議事務局, 70-76p.
- 田中昌一, 1972a: 標識放流結果からみた本邦太平洋沿岸のブリの回遊－Ⅰ. 日水誌, **38**, 29-32.
- 田中昌一, 1972b: 標識放流結果からみた本邦太平洋沿岸のブリの回遊－Ⅱ. 日水誌, **38**, 93-96.
- 田中昌一, 1973: 標識放流結果からみた本邦太平洋沿岸のブリの回遊－Ⅲ. 日水誌, **39**, 17-23.
- Tanaka S., 1978: Migration model and population dynamics of large-sized yellowtails in the Pacific Ocean along the Japanese coast inferred from tagging experiments－Ⅰ Same year recaptures. *Bull. Japan. Soc. Sci. Fish.*, **45**(3), 297-303.

- Tanaka S., 1984: Migration model and dynamics parameters of large-sized yellowtails in the Pacific Ocean along the Japanese coast inferred from tag recaptures after the year of release, *Bull. Japan. Soc. Sci. Fish.*, **50**(8), 1341-1347.
- 東海区水産研究所編, 1966: モジャコ採捕のブリ資源に及ぼす影響に関する研究. 東海区水産研究所, 99pp.
- 東海区水産研究所, 南西海区水産研究所編, 1970: モジャコ採捕のブリ資源に及ぼす影響に関する研究報告書(続報). 東海区水産研究所・南西海区水産研究所, 99pp.
- 辻 俊宏, 2000: 能登半島沿岸で漁獲されるブリ成魚の成熟度. 石川県水総セ研報, **2**, 37-39.
- 内山 勇, 1997: 日本海のブリ資源. 水産海洋研究, **61**, 310-312.
- 上原伸二, 三谷卓美, 石田実, 1996: ブリの加入量・加入前資源量の把握技術の開発. 平成7年度我が国周辺漁業資源調査・特定水産資源評価技術開発調査報告書, 水産庁・6水産研究所, 95-102.
- 上原伸二, 三谷卓美, 石田実, 1998: 東シチ海におけるブリの漁獲と産卵場. 南西外海の資源・海洋研究, **14**, 55-62.
- 内田恵太郎, 1954: 対馬暖流海域の浮遊魚卵, 魚稚仔附-ホンサバ, マアジ, ブリの卵乃至稚魚について. 対馬暖流開発調査研究報告, **1**, 水産庁, 111-115.
- 内田恵太郎, 1955: 対馬暖流海域における重要魚類稚仔の分布と出現期. 対馬暖流開発調査第3回シンポジウム発表論文, 水産庁, 333-335.
- 内田恵太郎, 道津喜衛, 水戸 敏, 中原官太郎, 1958a: ブリの産卵および初期生活史. 九大農学部学芸雑誌, **16**, 329-342.
- 内田恵太郎, 道津喜衛, 水戸 敏, 中原官太郎, 1958b: ブリの産卵および初期生活史. ていち, **17**, 19-32.
- 内田恵太郎, 庄島洋一, 1958: 流れ藻に関する研究・流れ藻に伴う稚仔魚-I. 昭和32年度の津屋崎附近における調査. 日水誌, **24**, 411-415.
- 渡辺和春, 1979: 春・夏期に放流した標識魚の再捕結果からみた対馬暖流水域におけるブリの分布と回遊. 日水研報, **30**, 131-164.
- 山川文男, 1989: ブリ幼魚目視調査結果について. 第29回ブリ予報技術連絡会議議事録, 日本海ブリ予報技術研究チーム, **8**.
- 山寺敏之, 1935: 海洋歴の研究. 定置漁業界, **24**, 6-21.
- 吉田忠生, 1963: 流れ藻の分布と移動に関する研究. 東北水研報, **23**, 141-186.

Behaviour, Occurrence, and Aquatic Toxicity of New Antifouling Biocides and Preliminary Assessment of Risk to Aquatic Ecosystems

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Abstract Paints containing biocides prevent the adhesion of organisms to the hulls of ships, and are hazardous to aquatic ecosystems. I reviewed published literature reporting the behaviour, occurrence, and toxicity in aquatic environments of the organotin-free new antifouling biocides. Our analysis included the representative alternative new biocides; 2,4,5,6-tetrachloro-isophthalonitrile (chlorothalonil), N'-dimethyl-N-phenylsulphamide (dichlofluanid), 1-(3,4-dichlorophenyl)-3,3 dimethylurea (diuron), 2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine (Irgarol 1051), 4,5-dichloro-2-(n-octyl)-4-isothiazoline (Sea Nine 211), 2-(thiocyanomethylthio)benzothiazole (TCMTB), bis-(1-hydroxy-2(1H)-pyridinethionate-O,S) zinc (zinc pyrithione, ZnPT), zinc ethylene bis-(dithiocarbamate) (zineb), pyridinetriphenylboron (PK), and bis-(1-hydroxy-2(1H)-pyridinethionate-O,S) copper (copper pyrithione, CuPT). These new biocides are classified as "toxic to very toxic" according to guidelines of the Organization for Economic Co-operation and Development, with toxicity values similar to those of TBT. The new biocides are decomposed by hydrolysis, photolysis, and biological activity and thus are less persistent in the environment than TBT, but low vapour pressure limits their evaporation. Because of medium octanol-water coefficient (K_{ow}) and organic carbon partitioning coefficient (K_{oc}) values, these new biocides, except for zineb, ZnPT, and CuPT, tend to be distributed in both seawater and sediments but have low bioaccumulation in aquatic organisms. Chlorothalonil, dichlofluanid, diuron, Irgarol 1051 and its degradation product, 2-methylthio-4-tert-butylamino-6-amino-s-triazine (M1), and Sea Nine 211 have been found in natural waters, with higher concentrations in marinas and small harbours for fishing boats than in coastal waters, but insufficient data are available to assess contamination by these biocides. The reported concentrations of Irgarol 1051, M1, and Sea Nine 211 exceed their respective predicted no-effect concentration values, especially in marinas and fishery harbours, suggesting that these new biocides are already harming aquatic ecosystems in some areas. Further research is needed to assess the ecotoxicological risks of these biocides and to develop methods for estimation of their concentrations in ambient water.

Key words : TBT-free antifouling paint, biocide, fate in environment, toxicity, risk assessment, aquatic organisms

Introduction

Prevention of biofouling, the adhesion of organisms

to the hulls of ships, is a crucial aspect of ship maintenance and the effective operation of marine vessels. Many antifouling paints have been developed

and widely used. Cuprous oxide-based paints were used until around 1965, when they were replaced with paints containing organotin compounds, most commonly tributyltin (TBT) or triphenyltin (TPT), which effectively control biofouling by a wide variety of organisms. It has become apparent, however, that organotin compounds are hazardous to aquatic environments. These organotin compounds are poorly biodegradable and thus remain in the environment for a long time. Moreover, these compounds are highly toxic to aquatic organisms, so bioaccumulation is a concern.

In Japan, the use of organotin compounds in antifouling paints has been regulated by the Chemical Substances Control Law, which prohibits the manufacture, import, and sale of organotin compounds, and by notifications and other forms of administrative guidance together with controls on use that are voluntarily imposed by various organizations. The Marine Environment Protection Committee of the International Maritime Organization has been exploring a world-wide prohibition of the application of organotins as antifouling biocides and in October 2001 adopted the International Convention on the Control of Harmful Antifouling Systems on Ships, which resulted in a ban on the application of organotin-based antifouling paints. Therefore, a number of organotin-free antifouling paints have been developed. In addition to cuprous oxide, several organic chemical substances, such as 2-mercaptopyridine N-oxide zinc salt, are used as biocides in organotin-free antifouling paints. Few studies have been undertaken on either the fate in the aquatic environment or the harmful effects on aquatic organisms of these new antifouling biocides.

Author investigated ecotoxicological information available in published literature to estimate the harmful effects of these new biocides on aquatic organisms and the marine environment.

Physicochemical properties

Two studies (Thomas 2001a; Harino 2004) provided data on representative new biocides (Table 1). Thomas (2001a) listed 8 representative new biocides (Fig. 1), 2,4,5,6-tetrachloro-isophth

alonitrile (chlorothalonil), N'-dimethyl-N-phenylsulphamide (dichlofluanid), 1-(3,4-dichlorophenyl)-3,3-dimethylurea (diuron), 2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine (Irgarol 1051), 4,5-dichloro-2-(n-octyl)-4-isothiazoline (Sea Nine 211), 2-(thiocyanomethylthio)benzothiazole (TCMTB), bis-(1-hydroxy-2(1H)-pyridinethionate-O,S) zinc (zinc pyrithione, ZnPT), and zinc ethylene bis-(dithiocarbamate) (zineb). Reported values of octanol-water coefficient (K_{ow}), solubility, and estimated organic carbon partitioning coefficient (K_{oc}) for these compounds are summarized in Table 1. Harino (2004) summarized the physicochemical properties of the new biocides pyridinetriphenylboron (PK) and bis-(1-hydroxy-2(1H)-pyridinethionate-O,S) copper (copper pyrithione, CuPT) (Table 1). Most of these new biocides have been used as pesticides (herbicides and fungicides); however, Sea Nine 211, CuPT, and PK were produced for use as antifouling biocides (Fig. 1).

PK (Hokko Chemical Industry) was introduced commercially as an alternative biocide in 1993. Limited physicochemical data are available for this compound; therefore, it is difficult to assess its impact on aquatic environments.

Vapour pressures of chlorothalonil, dichlofluanid, Irgarol 1051, diuron, and Sea Nine 211 are 0.076 mPa (25 °C), 0.016 mPa (20 °C), 0.089 mPa, 0.41 mPa (50 °C), and 0.98 mPa (25 °C), respectively. These values are lower than that of TBTO, and indicating that these new biocides tend to mainly remain in the liquid phase.

Diuron is the most soluble of the new biocides (35 mg/L). The solubilities of Sea Nine 211, TCMTB, ZnPT, and Irgarol 1051 are 14 mg/L, 10.4 mg/L, 8 mg/L, and 7 mg/L, respectively; these compounds have greater solubility than chlorothalonil, dichlofluanid, and CuPT.

The log K_{ow} values of the new biocides ranged from 0.6 to 4.0. Chlorothalonil has the highest (log K_{ow} of 4.0), and zineb has the lowest (log K_{ow} of 0.6). In spite of the low solubilities of ZnPT and CuPT, both have low log K_{ow} values of 0.9 (Table 1), suggesting that these new biocides may not be bioconcentrated to high concentrations in aquatic organisms.

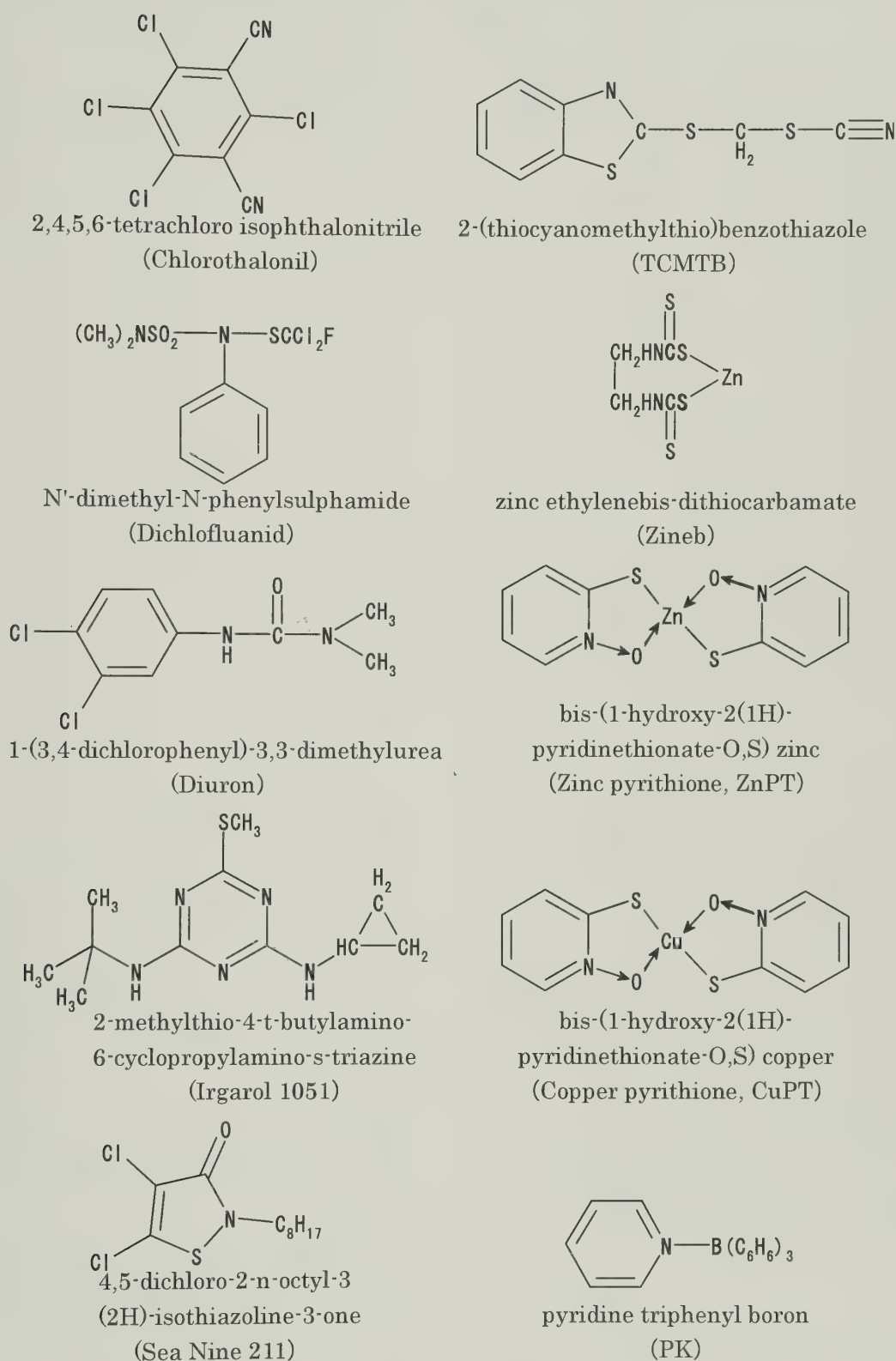


Fig. 1. The constitutional formula of representative new biocides.

Table 1. Physico-chemical properties of new biocides^{1*} and bis (tributyltin) oxide (TBTO)^{2*}

Biocide	Solubility (mg/L)	LogKow	Estimated LogKoc ^{3*}	Vapour Pressure (mPa)
Chlorothalonil	0.9	4.0	3.8	0.076(25°C)
Dichlofluanid	1.3	3.7	3.5	0.016(20°C)
Diuron	35	2.8	2.6	0.41(50°C)
Irgarol 1051	7	3.95	3.7	0.089
Sea Nine 211	14	2.8	2.6	0.98(25°C)
TCMTB	10.4	3.3	3.1	—
Zineb	0.07-10	0.8	0.6	—
ZnPT	8	0.9	0.7	—
CuPT	<1	0.9	0.7	—
PK	1	—	—	<133(20°C)
TBTO	<1—>100	3.19—3.84	2.98—3.63	1.0(20°C)

^{1*}: Reported by Thomas (2001a) and Harino (2004).

^{2*}: Reported by UNEP, ILO and WHO(1990)

^{3*}: Koc was estimated by using the equation ($\log Koc = \log Kow - 0.21$) presented by Karickhoff (1981).

Log Koc values estimated by the equation ($\log Koc = \log Kow - 0.21$) (Karickhoff, 1981) are also shown in Table 1. Log Koc values of chlorothalonil, dichlofluanid, diuron, Irgarol 1051, Sea Nine 211, and TCMTB ranged from 2.9 to 3.8, and were same order of TBTO. The log Koc values of zineb (0.6), ZnPT (0.7), and CuPT (0.7), were comparatively low. The medium Koc values of the new biocides such as chlorothalonil suggest that these biocides as well as TBTO may exist in both water and sediments; whereas biocides with low Koc values, such as zineb, ZnPT, and CuPT, may exist mainly in water. Galvin *et al.* (1998) reported that ZnPT and CuPT exist in sediments as copper and manganese complexes.

Degradation

Author reviewed degradation processes, including photolysis, hydrolysis, and biodegradation, for the new biocides.

Chlorothalonil

Chlorothalonil is rapidly photodegraded by sunlight, with a half-life ranging from 1 to 48 h according to photodegradation measurements in several natural waters. Chloro-1,3-dicyanobenzene and benzamide are produced as photodegradation products (Sakkas *et al.*, 2002a). Biodegradation was found to be slower than photodegradation, with a half-life of 8 to 9 days (Walker *et al.* 1988).

Dichlofluanid

Thomas (2001a) reviewed the degradation of dichlofluanid in the natural environment, and reported a half-life of 18 h, which is relatively rapid degradation.

Diuron

Harino (2004) reported that no half-life data were available on diuron. Ellis and Camper (1982) reported that more than 80% of diuron was degraded in seawater within 27 days, with most of the degradation due to biological activity; photodegradation had only a minor effect. Under aerobic conditions, the primary degradation products of diuron are 3-(3,4-dichlorophenyl)-1-methyl urea,

3,4-dichlorophenyl urea, and 3-(3,4-dichloroaniline), whereas the primary anaerobic degradation product is 3-(4-chlorophenyl)-1,1'-dimethylurea.

Irgarol 1051

Many researchers (Liu *et al.*, 1997; Okamura, 2002a; Okamura and Sugiyama, 2004) have studied the degradation of Irgarol 1051. Liu *et al.* (1997) demonstrated that Irgarol 1051 is degraded by white rot fungus (*Phanerochaete chrysosporium*) to 2-methylthio-4-tert-butylamino-6-amino-s-triazine (M1) through N-dealkylation of a cyclopropyl group from the cyclopropylamino side-chain at the 6-position of the s-triazine ring. M1 is not degraded further by *P. chrysosporium*, and appears to accumulate as an end degradation product in aquatic environments. Irgarol 1051 is also transferred to M1 by Hg-catalyzed hydrolysis (Liu *et al.*, 1999a) and by photolysis (Okamura, 2002a). The photodegradation of Irgarol 1051 is stimulated by dissolved organic materials such as humic and fulvic substances (Okamura and Sugiyama, 2004). The Irgarol 1051 half-life (Harino 2004) ranged from 36 to 273 days. These results suggest that Irgarol 1051 is fairly stable in aquatic environments.

Sea Nine 211

Sea Nine 211 undergoes hydrolysis, photolysis, and biodegradation. Shade *et al.* (1993) reported that Sea Nine 211 is stable in sterile water at pH 7 but is hydrolyzed under acidic and alkaline conditions. Harino (2004) reported that biodegradation is rapid in seawater, with a half-life ranging from <0.5 to >30 days; N-octyl ozamic acid, 4,5-dichlorothiazole, and N-octyl carbamic acid are produced as the primary degradation products.

TCMTB

Laboratory results by Brownlee *et al.* (1992) indicated that TCMTB is rapidly degraded into 2-mercaptobenzothiazole and 2-(methylthio) benzothiazole. TCMTB is easily degraded by photolysis, with a reported half-life of <0.5 h, and has a reported biodegradation half-life of 740 h in natural waters.

Zineb

Zineb is known to degrade rapidly through hydrolysis to 5,6-dihydro-3H-imidazo-(2,1-c)-1,2,4-dithiazole-3-thion, ethylene diisothiocyanate, and ethylenethiourea (Hunter and Evans, 1991).

A half-life of 96 h was observed in hydrolysis experiments at pH 10 and 20 °C.

ZnPT and CuPT

Comprehensive studies on degradation of ZnPT and CuPT (Turley *et al.* 2000) found that the hydrolysis half-life of CuPT in sterilized artificial seawater is 12.9 days. Results of a seawater die-away experiment indicated that at an initial CuPT concentration of 52 µg/L, the half-life with respect to biodegradation is approximately 4 days in seawater and 7 h in river water. CuPT is also sensitive to light, with a reported photolysis half-life of 29.1 min.

The reported hydrolysis half-life of ZnPT is >90 days, and ZnPT also biodegrades in seawater, with a half-life of approximately 4 days. Like CuPT, ZnPT has high sensitivity to light; its reported half-life is 17.5 min under a filtered xenon arc lamp and <2 min under natural sunlight.

These results suggest that ZnPT and CuPT are mainly degraded by photolysis in aquatic environments. Pyridine sulfonic acid is produced from ZnPT and CuPT as a degradation products.

PK

There are no available data on PK hydrolysis or biodegradation in the aquatic environment. Photodegradation of PK dissolved in artificial seawater and subjected to UV radiation (254 nm, 5.5 W) (Amey and Waldron, 2004) was very rapid and in <1 h led to boric acid production by elimination of pyridine and phenol.

In summary, the information on the degradation of these new antifouling biocides suggests that these new biocides are not stable compared with the previously used TBT. Because of their more rapid degradation, these new biocides pose less of an environmental risk than TBT.

Concentrations in the Aquatic Environment

Chlorothalonil

Chlorothalonil concentrations in seawater were studied in coastal areas of the United Kingdom (Thomas *et al.* 2002; Voulvoulis *et al.* 2000). Chlorothalonil was not detected in seawater from Southampton (Thomas *et al.*, 2002). In seawater of Blackwater Estuary, however, concentrations

<0.0002–0.00138 $\mu\text{g/L}$ were reported by Voulvoulis *et al.* (2000).

Dichlofluanid

Dichlofluanid was detected in coastal waters in Greece (Sakkas *et al.*, 2002b) and Spain (Martinez *et al.*, 2001) in concentrations ranging from <0.001 to 0.284 $\mu\text{g/L}$ and from <0.002 to 0.76 $\mu\text{g/L}$, respectively.

Diuron

The concentration of diuron in seawater was determined by Thomas *et al.* (2001b) at the south coast of the United Kingdom (Southampton and Sutton Harbour). In Southampton, the concentration in seawater ranged from 0.004 to 6.742 $\mu\text{g/L}$ in marinas and harbours and from <0.001 to 0.465 $\mu\text{g/L}$ in estuarine water. The concentrations in Sutton Harbour ranged from 0.001 to 0.334 $\mu\text{g/L}$. Harino *et al.* (2005) measured concentrations of diuron in seawater in Osaka Port, Japan, that ranged from 0.013 to 0.35 $\mu\text{g/L}$.

Irgarol 1051

The concentrations of Irgarol 1051 in the coastal waters of Japan, Canada, France, the United Kingdom, Germany, and Sweden and in the lakes of Switzerland are summarized in Table 2. Irgarol 1051 was not detected in seawater samples taken from 4 locations in Canada, including Vancouver. It was detected, however, in the coastal waters of France, the United Kingdom, Germany, Sweden, and Japan and in samples taken from marinas in the lakes of Switzerland.

The concentrations in seawater samples taken in France ranged from below the detection limit to 1.7 $\mu\text{g/L}$, with the highest values being measured for samples taken in the marinas of the Cote d'Azur region (Readan *et al.*, 1993). Irgarol 1051 was measured by Tolosa *et al.* (1996) at marinas, ports, and beaches; the concentrations were 0.022–0.64 $\mu\text{g/L}$, 0.0138–0.264 $\mu\text{g/L}$, and <0.0015–0.017 $\mu\text{g/L}$, respectively. The concentrations at marinas were higher than those at ports and beaches. The concentrations determined by Readan *et al.* (1993) did not differ from those reported by Tolosa *et al.* (1996). These results suggest that the annual variation of concentrations is not significant.

In a United Kingdom survey covering 7 coastal areas (Gough *et al.*; 1994; Zhou *et al.*; 1996; Scarlett

et al.; 1997; Thomas *et al.*, 2001b), the Irgarol 1051 concentration in seawater ranged from <0.001 to 0.682 $\mu\text{g/L}$. The concentrations were higher in the marinas than in coastal waters. The highest concentration of 0.682 $\mu\text{g/L}$ was measured in a marina in Humber.

The Irgarol 1051 concentrations measured at marinas in the North Sea and the Baltic Sea were 0.011–0.17 $\mu\text{g/L}$ and 0.08–0.44 $\mu\text{g/L}$, respectively (Biselli *et al.*, 2000). In general, concentrations were higher in the Baltic Sea than in the North Sea. The concentrations measured in marinas in Germany were similar to those in the marinas of France and the United Kingdom.

The Irgarol 1051 concentrations from 0.03 to 0.4 $\mu\text{g/L}$ were reported at marinas in estuarine areas of Sweden. This substance has been detected not only in seawater but in terrestrial water samples taken from marinas in the lakes of Switzerland.

In Japan, Okamura *et al.* (2000a) and Liu *et al.* (1999b) conducted surveys of environmental contamination in the Seto Inland Sea. Irgarol 1051 and its degradation product M1 were detected in the seawater but not in sediment. Irgarol 1051 concentrations in seawater ranged from below the detection limit to 0.296 $\mu\text{g/L}$, with the higher concentrations being detected in marinas and small fishing ports located in the prefectures of Hiroshima, Okayama, Hyogo, and Kagawa (Table 5). M1 has been detected in seawater taken in the Seto Inland Sea, and M1 concentrations in seawater ranged from below the detection limit to 1.27 $\mu\text{g/L}$. These results indicate that M1 concentrations tend to be higher than those of Irgarol 1051 and that M1 remains in aquatic environments for a longer period than Irgarol 1051.

Irgarol 1051 concentrations in seawater were found to be higher in marinas and small fishing ports where pleasure boats and small fishing vessels are moored than in large ports used by ocean-going vessels. Irgarol 1051 concentrations are also higher in Europe (France, the United Kingdom, Germany, and Sweden) than in Japan. These results indicate that antifouling paints containing Irgarol 1051 are predominantly used on pleasure boats and small fishing vessels, and they are used in greater volumes in the European countries than in Japan.

Table 2. Concentration of Irgarol 1051 and its degradation products (M1) in water at various regions.

Country and Location		Concentration (μ g/L)		Reference
		Irgarol 1051	M1	
Japan				
Seto Inland Sea	marina	nd – 0.0853	0.0288 – 1.27	Okamura <i>et al.</i> (2000a)
	fishery harbour	nd – 0.296	nd – 1.21	Okamura <i>et al.</i> (2000a)
	Kojima port	nd – 0.0195		Liu <i>et al.</i> (1999b)
	Kobe port	nd		Liu <i>et al.</i> (1999b)
Canada				
Vancouver, Tront	marina	nd		Liu <i>et al.</i> (1999b)
Montreal & Halifax	port	nd		
France				
Coate d'Azur	marina	0.11 – 1.7		Readan <i>et al.</i> (1993)
	port	<0.005 – 0.28		
	marina	0.022 – 0.64		Tolosa <i>et al.</i> (1996)
	port	0.0138 – 0.264		
	beach	<0.0015 – 0.017		
United Kingdom				
Hamble estuary		0.012 – 0.19		Gough <i>et al.</i> (1994)
Medway estuary		0.004 – 0.018		
NRA southern region	marina	0.052 – 0.5		
	estuary	<0.002 – 0.014		
	river	<0.002		
Humber	marina	0.169 – 0.682		Zhou <i>et al.</i> (1996)
Plymouth	marina	0.028 – 0.127		Scarlett <i>et al.</i> (1997)
Southampton	marina & harbour	0.007 – 0.403		Thomas <i>et al.</i> (2001b)
	estuary water	<0.001 – 0.047		
Sutton harbour		<0.001 – 0.084		
Germany				
North Sea	marina	0.011 – 0.17		Biselli <i>et al.</i> (2000)
Baltic Sea	marina	0.08 – 0.44		
Sweden				
Fiskebackski	marina	0.03 – 0.4		Tolosa <i>et al.</i> (1996)
Switzerland				
Port d'Ouchy	marina	0.0025 – 0.145		Toth <i>et al.</i> (1996)

Irgarol 1051: 2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine
M1: 2-methylthio-4-t-butylamino-6-amino-s-triazine
nd: below the detection limit

Irgarol 1051 concentrations in sediments were measured by Biselli *et al.* (2000) in the North Sea and Baltic Sea and by Albanis *et al.* (2002) in the coastal area of Greece. The concentrations ranged from 3 to 25 $\mu\text{g/kg}$ dry weight in the North Sea, 4 to 220 $\mu\text{g/kg}$ dry weight in the Baltic Sea, and less than the detection limit to 338 $\mu\text{g/kg}$ dry weight in Greece. Concentrations were particularly high in the sediments of marinas, a tendency similar to that observed in seawater.

Sea Nine 211

Thomas *et al.* (2001b) reported that Sea Nine 211 was not detected in seawater and sediments collected at marinas on the south coast of the United Kingdom in 1998. On the other hand, Martinez *et al.* (2001) reported that Sea Nine 211 was detected at concentrations ranging from 0.26 to 3.7 $\mu\text{g/L}$ in seawater from the Spanish Mediterranean. Sea Nine 211 was detected in the coastal areas of Greece at concentrations ranging from <0.0063 to 0.049 $\mu\text{g/L}$ (Sakkas *et al.*, 2002b) and in Osaka Port in Japan (Harino *et al.*, 2005) from <0.0003 to 0.0055 $\mu\text{g/L}$.

TCMTB, ZnPT, CuPT, and PK

Thomas *et al.* (2004) reported that their monitoring studies did not detect chlorothalonil, dichlofluanid, ZnPT, Sea Nine 211, or TCMTB in seawater in the United Kingdom. Harino *et al.* (2005) also reported that ZnPT and CuPT were not detected in seawater collected in Osaka Port, Japan. I found no literature reporting concentration of PK in aquatic environments.

Harmful effects of new biocides on aquatic organisms

Chlorothalonil

Acute toxicity of chlorothalonil to *Daphnia magna*, *Onchorhynchus mykiss*, and *Gasterosteus aculeatus* are summarized in Table 3. The 48 h EC₅₀ to *D. magna* ranged from 97 to 129 $\mu\text{g/L}$, and the 96 h LC₅₀ to fish species ranged from 69 to 76 $\mu\text{g/L}$. Acute toxicities to crustacean and fish species are significant, and are "very toxic" according to Organization for Economic Co-operation and Development (OECD) ecotoxicity classification guidelines (OECD, 1996).

Sea Nine 211

Sea Nine 211 is acutely toxic to phytoplankton at concentrations of 13.9–32 $\mu\text{g/L}$, to crustacean species at concentrations of 4.7–1,312 $\mu\text{g/L}$, to bivalves at a concentration of 850 $\mu\text{g/L}$, and to fish species at concentrations of 2.7–20.5 $\mu\text{g/L}$ (Table 3). From these data, author conclude that this compound exhibits weaker acute toxicity to bivalves than other aquatic organisms such as phytoplankton and fish. Based on these acute toxicity values, Sea Nine 211 is classified as "very toxic to toxic" according to the OECD guidelines.

Chronic toxicities of Sea Nine 211 to *D. magna* and sheepshead minnows (*Cyprinodon variegatus*) are reported to occur at concentrations of 1.2 $\mu\text{g/L}$ and 6 $\mu\text{g/L}$, respectively (Table 4). The 28 d LC₅₀ to rainbow trout larvae is 14 $\mu\text{g/L}$ (Okamura, *et al.*, 2002), leading to fears that the chronic toxicity of Sea Nine 211 is equivalent to that of other antifouling compounds such as ZnPT, CuPT, and PK.

ZnPT and CuPT

Published literature contains very little data on the acute toxicity of ZnPT and CuPT to aquatic organisms (Table 3). The respective toxicity values of ZnPT are reported to be 2.06–28 $\mu\text{g/L}$ for phytoplankton (Shipbuilding Research Association of Japan, 2002), 29–34 $\mu\text{g/L}$ for *D. magna* (Shipbuilding Research Association of Japan, 2002), and 2.6–400 $\mu\text{g/L}$ for fish species (Shipbuilding Research Association of Japan, 2002). CuPT exposure causes acute toxicity leading to decreased phytoplankton growth at concentrations of 2.8–35 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 2002), *D. magna* immobilization at 22 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 2002), and decreased survival of fish species at 4.3–7.67 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 2002). Mochida *et al.* (2006) recently studied the acute toxicity of CuPT and ZnPT to toy shrimp (*Heptacarpus futilirostris*) and red sea bream (*Pagrus major*); 96 h LC₅₀s to red sea bream were 9.3 $\mu\text{g/L}$ for CuPT and 98.2 $\mu\text{g/L}$ for ZnPT. On the other hand, 96 h LC₅₀s of CuPT and ZnPT were 2.5 and 120 $\mu\text{g/L}$, respectively, for toy shrimp. Although the available toxicity data are limited, they indicate that CuPT exhibits greater acute toxicity to fish and shrimp species than does ZnPT. Toxicological data on ZnPT and CuPT evaluated on the basis of the OECD

classification guidelines correspond to the “very toxic” definition, suggesting that both compounds are highly toxic to aquatic organisms.

In organotin-free antifouling paints, the pyrrithione compounds (ZnPT and CuPT) are usually used in combination with cuprous oxide, and their acute toxicity may change with the presence of copper.

Mochida *et al.* (2006) examined the toxicities of pyrrithione compounds (ZnPT and CuPT) in the presence of copper ions. The presence of Cu²⁺ did not change the acute toxicity of CuPT but significantly increased the acute toxicity of ZnPT. According to Mochida *et al.* (2006), the enhancement of toxicity by Cu²⁺ is attributable to

Table 3. Acute toxicity of new biocides to aquatic organisms

Aquatic organisms	Endpoint	Acute toxicity					
		ZnPT ^{1*} (μ g/L)	CuPT ^{2*} (μ g/L)	Pyridine sulfonic acid(μ g/L)	PK ^{3*} (μ g/L)	Sea Nine 211 ^{4*} (μ g/L)	Chlorothalonil ^{5*} (μ g/L)
Micro algae							
<i>Selenastrum capricornutum</i>	72·120hEC50	28(2) ^{6*}	35(2)			32(4)	
<i>Skeletonema costatum</i>	72hEC50	2.06(2)	28.4(2)		2.15(1)	13.9(4)	
freshwater algae	120hLC50			28,900(2)			
Crustacean							
<i>Daphnia magna</i>	48hEC50	29–34(2)	22(2)	>122,000(2)			97–129(5)
mysid shrimp	96hLC50	6.3(2)		71,600(2)		4.7(4)	
toy shrimp	96hLC50	120(6)	2.5(6)				
brown shrimp	96hLC50					12.4(4)	
<i>Uca pugilator</i>	96hLC50					1,312(4)	
<i>Penaeus japonicus</i>	96hLC50	1,780(2)	43.6(2)		149(1)	12.6(1)	
<i>Tigriopus japonicus</i>	24hEC50	160(3)	31(3)		16(3)	30(2)	
<i>Tigriopus japonicus</i>	24hLC50	>500(3)	41(3)		110(3)	77(2)	
Mussel							
bay mussel	96hLC50					850(4)	
Fish							
<i>Lepomis macrochirus</i>	96hLC50					14(4)	
<i>Pimephales promelas</i>	96hLC50	2.6(2)	4.3(2)	68,500(2)			
<i>Oncorhynchus mykiss</i>	96hLC50	3.2(2)		57,100(2)		2.7(4)	69–76(5)
<i>Cyprinodon variegatus</i>	96hLC50	400(2)		>127,000(2)		20.5(4)	
<i>Pagrus major</i>	96hLC50	273(2)	7.67(2)		242(1)		
<i>Pagrus major</i>	96hLC50	98.2(6)	9.3(6)				
<i>Gasterosteus aculeatus</i>	96hLC50						73(5)

1*: Bis-(1-hydroxy-2(1H)-pyridinethionate-O,S)zinc, 2*: Bis-(1-hydroxy-2(1H)-pyridinethionate-O,S)copper,
3*: Pyridinetriphenylboron, 4*: 4,5-dichloro-2-n-octyl-3-isothiazolone(Sea Nine 211), 5*: 2,4,5,6-tetrachloro-isophthalonitrile
6*: Figure in the parenthesis exhibit the reference. (1): Shipbuilding Research Association of Japan (1999), (2) Shipbuilding Research Association of Japan (2001), (3): Shipbuilding Research Association of Japan (2002), (4): Shade *et al.* (1994), (5):Ernst *et al.* (1991), (6):Mochida *et al.* (2006)

Table 4. Chronic toxicity of new biocides to aquatic organisms

Aquatic organisms	Endpoint	Chronic toxicity				Reference
		ZnPT ^{1*} (μ g/L)	CuPT ^{2*} (μ g/L)	PK ^{3*} (μ g/L)	Sea Nine 211 ^{4*} (μ g/L)	
Crustacean						
<i>Daphnia magna</i>	21day chronic EC50				1.2	Shade <i>et al.</i> (1993)
Fish						
<i>Cyprinodon variegatus</i>	early life NOEC				6	Shade <i>et al.</i> (1993)
<i>Oncorhynchus tshawytscha</i>	7dLC50	8.4	7.6	140	14	Okamura <i>et al.</i> (2002b)
	14dLC50	5.6	3.0	84	14	
	21dLC50	4.9	1.7	61	14	
	28dLC50	4.6	1.3	42	14	

1*: Bis-(1-hydroxy-2(1H)-pyridinethionate-O,S)zinc, 2*: Bis-(1-hydroxy-2(1H)-pyridinethionate-O,S)copper,
3*: Pyridinetriphenylboron, 4*:4,5-dichloro-2-n-octyl-3-isothiazolone (Sea Nine 211)

conversion of ZnPT to more toxic CuPT.

ZnPT has significant effects on the embryogenesis of fish species, and an early life-stage toxicological study in which fertilized eggs were exposed to ZnPT confirmed deformities in the spinal curvature of fish larva after hatching (Goka, 1999). For rainbow trout larvae, Okamura *et al.* (2002b) obtained 28 d LC50 values of 4.6 $\mu\text{g/L}$ for ZnPT and 1.3 $\mu\text{g/L}$ for CuPT (Table 4). These toxicity values raise the concern that both compounds may exhibit high chronic toxicity to fish species.

ZnPT and CuPT are degraded in the aquatic environment to pyridine sulfonic acid. The acute toxicities of pyridine sulfonic acid were as follows: 120 h LC50 to freshwater algae was 28,900 $\mu\text{g/L}$, 48 h EC50 to *D. magna* was >122,000 $\mu\text{g/L}$, and 96 h LC50 to fish species was 57,100–>127,000 $\mu\text{g/L}$ (Table 3). The acute toxicities of pyridine sulfonic acid were weaker than those of the parent compounds, ZnPT and CuPT. These results suggest that toxicities of ZnPT and CuPT decrease in the process of degradation.

PK

Because of its recent development for use as a biocide in antifouling paints, only limited data are available on the harmful effects of PK on aquatic organisms (Table 3): the 72 h EC50 to the phytoplankton *Skeletonema costatum* is 2.15 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 1999), the 96 h LC50 to shrimp (*Penaeus japonicus*) is 149 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 1999), and the 96 h LC50 to red sea bream (*Pagrus major*) is 242 $\mu\text{g/L}$ (Shipbuilding Research Association of Japan, 1999). Although the acute toxicity of PK is weaker than that of ZnPT and CuPT, it is classified as “very toxic” according to the OECD toxicity guidelines.

The 28 d LC50 of PK is 42 $\mu\text{g/L}$ based on 28-day rearing experiments using rainbow trout larvae (Okamura, *et al.*, 2002). This value is equivalent to that of ZnPT and CuPT. These results raise a great concern of this compound’s harmful effect on aquatic organisms.

Irgarol 1051

Acute toxicity of Irgarol 1051 and its degradation product M1 to various aquatic organisms are summarized in Table 5. Irgarol 1051 does not affect

bacterial growth even at a concentration of 50,000 $\mu\text{g/L}$ (Okamura *et al.*, 2000a). The 24 h LC50 of Irgarol 1051 to crustacean species is reported to range from 5,700 to 12,000 $\mu\text{g/L}$ (Okamura *et al.*, 2000a). It inhibits mobilization (48 h EC50) at a concentration of 8,100 $\mu\text{g/L}$ (Ciba-Geigy, 1995). The 48 h LC50 to oyster larvae is 3,200 $\mu\text{g/L}$, whereas the 96 h LC50 to fish species ranges from 790 $\mu\text{g/L}$ for rainbow trout to 4,000 $\mu\text{g/L}$ for zebrafish (Hall Jr. *et al.*, 1999).

The EC50 for inhibition of phytoplankton growth ranges from 0.1 to 2.3 $\mu\text{g/L}$. Irgarol 1051 is very toxic to seaweed; germination and development of spores is inhibited at concentrations ranging from 0.6 to 5.9 $\mu\text{g/L}$ (Okamura *et al.*, 2000b). The constitutional formula of Irgarol 1051 resembles that of triazine herbicides; therefore, it seems reasonable that Irgarol 1051 is remarkably more toxic to aquatic vegetation such as phytoplankton and seaweed than to marine animals such as crustaceans and fish.

Based on the ecotoxicity classification guidelines (OECD, 1996), Irgarol 1051 acute toxicity values to phytoplankton and aquatic animals (crustaceans and fish) correspond to the definition of “very toxic” and “toxic”, respectively.

As shown in Table 5, the 72 h EC50 of M1 to phytoplankton growth ranged from 1.9 to 46 $\mu\text{g/L}$ (Okamura *et al.*, 2000a). The 96 h EC50 of M1 to the germination and development of the spores of seaweed ranged from 1.7 to 130 $\mu\text{g/L}$ (Okamura *et al.*, 2000b), whereas it is acutely toxic to crustacea (*Daphnia magna*) species at concentrations of 11,000 $\mu\text{g/L}$ (Okamura *et al.*, 2000a). EC50 and LC50 values of M1 are 10 times greater than those of the parent compound. Although the toxicity of Irgarol 1051 decreases as the compound is degraded, its degradation product M1 is classified as “toxic” according to the OECD ecotoxicity classifications.

The chronic toxicity of Irgarol 1051 was evaluated by an early life-stage toxicity test using rainbow trout (embryo to larval stage) (Table 6). A no observed effect concentration of 4.02 $\mu\text{g/L}$ was determined by the inhibition of larval growth during 60 days after hatching (Hall Jr. *et al.*, 1999). Chronic toxicity and acute toxicity values revealed that the acute/chronic toxicity ratios of Irgarol 1051 to rainbow trout ranged from 3 to 100. This

Table 5. Acute toxicity of Irgarol 1051 and its biodegradation products (M1) to aquatic organisms

Aquatic organisms		Endpoint	Toxicity index	Acute toxicity(μ g/L)		Reference
Class	Species			Irgarol 1051 1*	M1 2*	
Bacteria	<i>Vibrio fischeri</i>	bioluminescence	30min.EC50	>50,000	>50,000	Okamura <i>et al.</i> (2000a)
Micro algae	<i>Selenastrum capricornutum</i>	biomass	72hEbC50	1.6	19	Okamura <i>et al.</i> (2000a)
	<i>Selenastrum capricornutum</i>	growth rate	72hErC50	2.3	46	Okamura <i>et al.</i> (2000a)
	<i>Senedesmus</i> sp.		72hEC50	1.4		Ciba-Geigy (1995)
	<i>Raphidocelis subcapitata</i>		EC50	1		Ciba-Geigy (1995)
	<i>Anabaena flos-aquae</i>		EC50	2		Ciba-Geigy (1995)
	<i>Navicula pelliculata</i>		EC50	0.1		Ciba-Geigy (1995)
	<i>Skeletonema costatum</i>		EC50	0.4		Ciba-Geigy (1995)
Seaweed	<i>Porphyra yezoensis</i>	conchospore survival	96hLC50	5,000	6,500	Okamura <i>et al.</i> (2000b)
		conchospore germination	96hEC50	4.1	130	Okamura <i>et al.</i> (2000b)
		conchospore growth	96hEC50	0.6	17	Okamura <i>et al.</i> (2000b)
	<i>Eisenia bicylis</i>	gamatephyte growth	96hEC50	5.9	>32	Okamura <i>et al.</i> (2000b)
Crustacean	<i>Daphnia magna</i>	lethality	24hLC50	16,000	17,000	Okamura <i>et al.</i> (2000a)
			48hLC50	8,300	11,000	Okamura <i>et al.</i> (2000a)
	<i>Daphnia pulex</i>	lethality	24hLC50	5,700	27,000	Okamura <i>et al.</i> (2000a)
	<i>Thamnocepharus platyurus</i>	lethality	24hLC50	12,000	19,000	Okamura <i>et al.</i> (2000a)
	<i>Daphnia magna</i>		48hEC50	8,100		Ciba-Geigy (1995)
	<i>Myxidopsis bahia</i>	lethality	96hLC50	40		Hall Jr. <i>et al.</i> (1999)
	<i>Artemia salina</i>	lethality	24hLC50	>4,000	>4,000	Okamura <i>et al.</i> (2000a)
Bivalves	oyster larvae	lethality	48hLC50	3,200		Ciba-Geigy (1995)
Fish	<i>Cyprinodon variegatus</i>	lethality	96hLC50	3,500		Hall Jr. <i>et al.</i> (1999)
	<i>Barachydanio rerio</i>	lethality	96hLC50	4,000		Ciba-Geigy (1995)
	<i>Oncorhynchus mykiss</i>	lethality	96hLC50	790		Hall Jr. <i>et al.</i> (1999)
	<i>Menidia beryllina</i>	lethality	96hLC50	1,580		Hall Jr. <i>et al.</i> (1999)
	<i>Lepomis macrochirus</i>	lethality	96hLC50	2,600		Hall Jr. <i>et al.</i> (1999)

1*:2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine, 2*: 2- methylthio-4-t-butylamino-6-amino-s-triazine

Table 6. Chronic toxicity of Irgarol 1051 to shrimp and fish.

Aquatic organisms		Endpoint	Exposure period	Chronic toxicity (μ g/L)	Reference
Class	Species				
Crustacean	<i>Myxidopsis bahia</i>	growth	28day	110 (LOEC: 260)	Hall Jr. <i>et al.</i> (1999)
	shrimp	survival	28day	260 (LOEC: 490)	Hall Jr. <i>et al.</i> (1999)
Fish	<i>Oncorhynchus mykiss</i>	hatchability		184	Hall Jr. <i>et al.</i> (1999)
		survival	60day after hatch	184	Hall Jr. <i>et al.</i> (1999)
		growth	60day after hatch	4.02 (LOEC:9.14)	Hall Jr. <i>et al.</i> (1999)
		reproduction	28day	260 (LOEC: 490)	Hall Jr. <i>et al.</i> (1999)
	<i>Oncorhynchus tshawytscha</i>	LC50	28day	88	Okamura <i>et al.</i> (2002b)

LOEC: lowest observed effect concentration
Irgarol 1051: 2-methylthio-4-t-butylamino-6-cyclopropylamino-s-triazine

ratio is similar to that of numerous other chemical substances such as lindane, PCP, DDT, and dieldrin. In summary, despite the paucity of data on the toxicity of the new antifouling biocides to aquatic organisms, available published data suggest that Irgarol 1051 is toxic to phytoplankton and seaweed and that CuPT and ZnPT are highly toxic to marine animals. The acute toxicity of these new biocides is found to be in the order of micrograms per liter,

which is equivalent to that of the TBT (Koyama and Shimizu, 1992; Horiguchi and Shimizu, 1992; IMO, 1997).

Preliminary risk assessment

The predicted no effect concentration (PNEC) was tentatively estimated from the lowest acute toxicity value by the OECD method. In accordance

with OECD method, the uncertainty factor of 100 was used to calculate PNEC. The values of PNEC for several new biocides are shown in Table 7 together with the observed concentrations of these biocides in natural waters.

The highest concentration found in natural water (EC) and PNEC of chlorothalonil, was 0.00138 $\mu\text{g/L}$ and 0.69 $\mu\text{g/L}$, respectively. EC / PNEC ratio was 0.002, and EC was two orders of magnitude lower than the PNEC value. Although the available data on chlorothalonil concentrations in natural waters are very limited, these results suggest that its hazardous impact on the aquatic environments can be considered small.

The PNEC was 0.016 $\mu\text{g/L}$ for Irgarol 1051, 0.19 $\mu\text{g/L}$ for M1, and 0.027 $\mu\text{g/L}$ for Sea Nine 211. The ECs of Irgarol 1051 ranged from 0.264 to 1.7 $\mu\text{g/L}$, and EC / PNEC ratio fell between 5.33 and 106.25. The EC was one or two orders magnitude higher than PNEC in marinas. The ECs of 1.21 and 1.27 $\mu\text{g/L}$ were observed in M1, and was EC / PNEC ratio was estimated as 6.37 or 6.68. The EC / PNEC ratio of Sea Nine 211 ranged from <0.01 to 137.

The higher EC / PNEC was recognized in marinas and fisheries harbours, raising concerns that these biocides are already harming the aquatic ecosystem, especially in marinas and fisheries harbours. The concentrations of Irgarol 1051, M1, and Sea Nine 211 in seawater collected at coastal areas such as beaches were significantly lower than PNEC values. Hazardous impacts by these new biocides seem restricted to particular areas such as marinas and fisheries harbours, where many pleasure boats and/or fishing boats are moored.

Harino *et al.* (2005) did not detect pyriithione compounds (ZnPT and CuPT) in the seawater of Osaka Port, Japan. These Osaka port results indicate that hazardous impact of pyriithione compounds are not yet a concern. The limited published data on the concentrations of PK preclude evaluation of the hazardous impact of PK on the marine ecosystem, and further studies are needed.

Conclusion

The low vapour pressure of the new biocides in

Table 7. Preliminary risk assessment of several new biocides to aquatic organisms by comparing PNEC with the highest concentration in natural waters.

Biocide		Lowest value of acute toxicity data ($\mu\text{g/L}$)	PNEC 1* ($\mu\text{g/L}$)	Highest concentration in natural water ($\mu\text{g/L}$) (EC)	EC / PNEC
Chlorothalonil	<i>D. magna</i> , <i>O. mykiss</i> ,	EC50 97 LC50 69	0.69	0.00138(Black water estuary)	0.002
Irgarol 1051	<i>S. Capricornutum</i> <i>D. magna</i> <i>O. mykiss</i>	EC50 1.6 EC50 8,100 LC50 790	0.016	0.0853(marina, Japan) 0.296(Fisheries harbour, Japan) 1.7(marina, France) 0.264(port, France) 0.682(marina, south coast of UK) 0.44(marina, the Baltic Sea) 0.4(marina, Sweden)	5.33 18.50 106.25 16.50 42.63 27.50 25.00
M1	<i>S. capricornutum</i> <i>D. magna</i>	EC50 19 EC50 27,000	0.19	1.27(marina, Japan) 1.21(fishery harbour, Japan)	6.68 6.37
Sea Nine 211	<i>S. capricornutum</i> mysid shrimp <i>O. mykiss</i>	EC50 32 LC50 4.7 LC50 2.7	0.027	0.26–3.7(Mediterranean Sea, Spain) <0.0063–0.049(Greece) <0.0003–0.00055(Osaka port, Japan)	9.62–137.00 <0.23–1.81 <0.01–0.02
Zn PT	<i>S. capricornutum</i> <i>D. magna</i> <i>P. promelas</i>	EC50 28 EC50 29 LC50 2.6	0.026	is not detected in Osaka port, Japan. The contamination is not exactly demonstrated.	—
Cu PT	<i>S. capricornutum</i> <i>D. magna</i> <i>H. futilirostris</i> <i>P. promelas</i>	EC50 35 EC50 22 LC50 2.5 EC50 4.3	0.025	is not detected in Osaka port, Japan. The contamination is not exactly demonstrated.	—
PK	<i>S. costatum</i> <i>T. japonicus</i> <i>P. major</i>	EC50 2.2 EC50 16 LC50 242	0.022	There are not available information on the concentration in seawater.	—

1*: PNEC was tentatively estimated from the lowest acute toxicity value by the OECD method using uncertainty factor of 100.

water limits their evaporation. Because of medium Kow and Koc values, the new biocides, except zineb, ZnPT, and CuPT, tend to be distributed in both seawater and sediments, and they are hardly bioconcentrated in aquatic organisms.

The new biocides are decomposed by hydrolysis, photolysis, and biological activity. Thus they are less stable than TBT and do not persist for a long time in aquatic environments. These new biocides are classified as “toxic” or “very toxic” by the OECD classification guideline, and show almost the same toxicity as TBT.

Chlorothalonil, dichlofluanid, diuron, Irgarol 1051 and its degradation product M1, and Sea Nine 211 have been detected in natural waters, with higher concentrations in marinas and small harbours for fishing boat than in coastal waters. Research on the occurrence in seawater in Japan was carried out only by Harino *et al.*, who did not detect pyrithione compounds. Detailed surveys are needed to clarify the present extent of contamination by the new biocides.

The reported concentrations of Irgarol 1051, M1, and Sea Nine 211 in natural waters exceed the PNEC values, particularly in marinas and fishery harbours. Igarol 1051, M1, and Sea Nine 211 are causing a hazardous impact on the aquatic ecosystem in marinas and fishery harbours. To assess the ecotoxicological risk of the new biocides in detail, further research on toxicity and the development of methods for estimating concentrations in natural waters are needed.

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References

- Albanis, T. A., Lambropoulou, D. A., Sakkas, U. A., and Konstantinou, I. K., 2002 : Antifouling paint booster biocide contamination in Greek marine sediments. *Chemosphere*, **48**, 475-485.
- Amey, R. L. and Waldron, C., 2004 : Efficacy and chemistry of BOROCIDETMP triphenyl boron-pyridine, a non-metal antifouling biocide. Proceedings of International Symposium on Antifouling Paint and Marine Environment (InSAfE), 234-243.
- Biselli, S., Bester, K., Huhnerfuss, H. and Fent, K., 2000 : Concentration of the antifouling compound Irgarol 1051 and of organotins in water and sediments of German North and Baltic Sea. *Mar. Pollut. Bull.*, **40**, 233-243.
- Brownlee, B. G., Carey, J. H., MacInnis, G. A. and Pellizzari, I. T., 1992 : Aquatic environmental chemistry of 2-(thiocyanomethylthio) benzothiazole and related benzothiazoles. *Environ. Toxicol. Chem.*, **11**, 1153-1168.
- Ciba-Geigy Co., 1995 : Irgarol 1051 in antifouling paints. Technical Information Bulletin.
- Ellis, P. and Camper, N., 1982 : Aerobic degradation of diuron by aquatic microorganisms. *J. Environ. Sci. Health*, **B17**, 277-288.
- Ernst, W., Doe, K., Jonah, P., Young, J., Julien, G. and Hennigar, P., 1991 : The toxicity of chlorothalonil to aquatic fauna and the impact of its operational use on a pond ecosystem. *Arch. Environ. Contam. Toxicol.*, **21**, 1-9.
- Galvin, R. M., Melland, J. M. R. and Montoya, M. R., 1998 : A contribution to the study of the natural dynamics of pyrithione (II), deactivation by direct chemical and absorptive oxidation. *Eur. Water Manage.*, **4**, 61-64.
- Goka, K., 1999 : Embryotoxicity of zinc pyrithione, an antidandruff chemical, in fish. *Environ. Res., Section A*, **81**, 81-83.
- Gough, M. A., Forthergill, J., Hendrie, J. D., 1994 : A survey of southern England coastal waters for the s-triazine antifouling compound Irgarol 1051. *Mar. Pollut. Bull.*, **28**, 613-620.
- Hall Jr., L. W., Giddings, J. M., Solomon, K. R., and Balcomb, R., 1999 : An ecological risk assessment for use of Irgarol 1051 as an algaecide for antifoulant paints. *Cri. Rev. Toxicol.*, **29**, 367-437.
- Harino, H., 2004 : Occurrence and degradation of representative TBT free-antifouling biocides in aquatic environment. *Coast. Mar. Sci.*, **29**, 28-39.

- Harino, H., Kitano, M., Mori, Y., Mochida, K., Kakuno, A. and Arima, S., 2005 : Degradation of the antifouling biocides in water. *J. Mar. Biol. Ass. U. K.*, **85**, 33-38.
- Horiguchi, T. and Shimizu, M., 1992 : Effects of organotin compounds on aquatic organisms, 7. Effects on aquatic organisms, mainly on mollusca. "Organotin Pollution and its Effects on Aquatic Organisms" (ed by Satomi, Y. and Shimizu, M.), Koseisha Koseikaku, Tokyo, pp. 99-135. (in Japanese).
- Hunter, J. E. and Evans, L. V., 1991 : Degradation of the biocide Zineb in marine culture medium. *Biofouling*, **3**, 113-137.
- IMO, 1997 : MEPC41/INF, Call for the worldwide ban on every use of organotin-based antifouling paints for ship bottom.
- Koyama, K. and Shimizu, A., 1992 : Effects of organotin compounds on aquatic organisms, 6. Effect on Fish, "Organotin Pollution and its Effects on Aquatic Organisms" (ed by Satomi, Y. and Shimizu, M.), Koseisha Koseikaku, Tokyo, pp. 86-98. (in Japanese)
- Liu, D., Maguire, R. J., Lau, Y. L., Pacepavicius, G. J., Okamura, H., Aoyama I., 1997 : Transformation of the new antifouling compound Irgarol 1051 by *Phanerochaete chrysosporium*. *Wat. Res.* **31**, 2363-2369.
- Liu, D., Pacepavicius, G. J., Maguire, R. J., Lau, Y. L., Okamura, H. and Aoyama, I., 1999a : Mercuric chloride catalyzed hydrolysis of the new antifouling compound Irgarol 1051. *Wat. Res.*, **33**, 155-163.
- Liu, D., Pacepavicius, G. J., Maguire, R. J., Lau, Y. L., Okamura, H., and Aoyama, I., 1999b : Survey for the occurrence of the new antifouling compound Irgarol 1051 in the aquatic environment. *Water Res.*, **33**, 2833-2843.
- Martinez, K., Ferrer, L., Hernando, M. D., Fernandez-Alba, A. R., Marce, R. M., Borull, F. and Balcelo, D., 2001 : Occurrence of antifouling biocides in the Spanish Mediterranean marine environment. *Environ. Technol.*, **22**, 543-552.
- Mochida, K., Ito, K., Harino, H., Kakuno, A. and Fujii, K., 2006 : Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (*Pagrus major*) and toy shrimp (*Heptacarpus futilirostris*). *Environ. Toxicol. Chem.*, **25**, 3058-3064.
- OECD, 1996 : Proposal for a Harmonized Classification System based on Acute Toxicity.
- Okamura, H., Aoyama, I., Liu, D., Maguire, R. J., Pacepavicius, G. J., and Lau, Y. L., 2000a : Fate and ecotoxicology of the new antifouling compound Irgarol 1051 in the aquatic environment. *Water Res.*, **34**, 3523-3530.
- Okamura, H., Aoyama, I., Takami, T., Maruyama, T., Suzuki, Y., Matsumoto, M., Katsuyama, I., Hamada, J., Beppu, T., Tanaka, O., Maguire, R. J., Liu, D., Lau, Y. L., and Pacepavicius, G. J., 2000b : Phytotoxicity of the new antifouling compound Irgarol 1051 and a major degradation product. *Mar. Pollut. Bull.*, **40**, 754-763.
- Okamura, H., 2002a : Photodegradation of the antifouling compounds Irgarol 1051 and Diuron released from a commercial antifouling paint. *Chemosphere*, **48**, 43-50.
- Okamura, H., Watanabe, T., Aoyama, I., and Hasobe, M., 2002b : Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells. *Chemosphere*, **46**, 945-951.
- Okamura, H. and Sugiyama, Y., 2004 : Photosensitized degradation of Irgarol 1051 in water. *Chemosphere*, **57**, 739-743.
- Readan, J. W., Kwing, L. L. W., Grondin, D., Bartocci, J., Villeneuve, J. P., and Mee, L. D., 1993 : Coastal water contamination from a triazine herbicide used in antifouling paints. *Environ. Sci. Technol.*, **27**, 1940-1942.
- Sakkas, V. A., Lambropoulou, D. A. and Albanis, T. A., 2002a : Study of chlorothalonil photodegradation in natural waters and in the presence of humic substances. *Chemosphere*, **48**, 939-945.
- Sakkas, V. A., Konstantinou, I. K., Lambropoulou, D. A. and Albanis, T. A., 2002b : Survey for the occurrence of antifouling paint booster biocides in the aquatic environment of Greece. *Environ. Sci. Pollut. Res.*, **9**, 327-332.
- Scarlett, A., Donkin, M. E., Fileman, T. W., and Donkin, P., 1997 : Occurrence of the marine antifouling agent Irgarol 1051 within the Plymouth sound locality : implications for the green macroalga *Enteromorpha*. *Mar. Pollut. Bull.*, **34**, 645-651.

- Shade, W. D., Hurt, S. S., Jacobson, A. H., and Reinert, K. H., 1993 Ecological risk assessment of a novel marine antifoulant. *Environ. Toxicol. Risk Assess.*, ASTM STP **1216**, 381-408.
- Shipbuilding Research Association of Japan, 1999 : Technical report on the prevention of marine pollution by antifouling paints -1997 edn, pp.17-53. (in Japanese)
- Shipbuilding Research Association of Japan, 2001 : Technical report on the prevention of marine pollution by antifouling paints -1999 edn, pp.48-87. (in Japanese)
- Shipbuilding Research Association of Japan, 2002. Technical report on the prevention of marine pollution by antifouling paints -2001 edn, pp.24-31. (in Japanese)
- Thomas, K. V. 2001a : The environmental fate and behaviour of antifouling paint booster biocides : a review. *Biofouling*, **17**, 73-86.
- Thomas, K. V., Fileman, T. W., Readman, J. W. and Waldock, M. J., 2001b : Antifouling paint booster biocides in the UK coastal environment and potential risks of biological effects. *Mar. Pollut. Bull.*, **42**, 677-688.
- Thomas, K. V., McHugh, M. and Waldock, M., 2002 : Antifouling paint booster biocides in UK coastal waters : input, occurrence and environmental fate. *Sci. Total Environ.*, **293**, 117-127.
- Thomas, K. V., Aldridge, J., Dyer, R., Hilton, M., McHugh, M., Reed, J., Reynolds, W. J. and Tolhurst, L., 2004 : The occurrence, fate and effects of selected antifouling paint booster biocides in UK docks, harbours and marinas. Proceedings of International Symposium on Antifouling Paint and Marine Environment (InSAfE), 177-195.
- Toth, S., Becker-van, Slooten, K., Spack, L., de Alencastro, L. F. and Tarradellas, J., 1996 : Irgarol 1051, an antifouling compound in freshwater, sediments, and biota of Lake Geneva. *Bull. Environ. Contam. Toxicol.*, **57**, 426-433.
- Tolosa, I., Readman, K. J. W., Billaevote, A., Ghilini, S., Bartocci, J., and Horvat, M., 1996 : Contamination of Mediterranean (Cote d'Azur) coastal waters by organotins and Irgarol 1051 used in antifouling paints. *Mar. Pollut. Bull.*, **32**, 335-341.
- Turley, P. A., Fem, R. J. and Ritter, J. C., 2000 : Pyrithiones as antifoulants : Environmental chemistry and preliminary risk assessment. *Biofouling*, **15**, 175-182.
- UNEP, ILO and WHO., 1990 : Environmental Health Criteria 116, Tributyltin Compounds, International Programme on Chemical Safety, Geneva, pp273.
- Voulvoulis, N., Scrimshaw, M. D. and Lester, J. N., 2000 : Occurrence of four biocides utilized in antifouling paints, as alternatives to organotin compounds, in water and sediments of a commercial estuary in the UK. *Mar. Pollut. Bull.*, **40**, 938-946.
- Walker, W. W., Cripe, C. R., Pritchard, P. H. and Bourquin, A. W., 1988 : Biological and biotic degradation of xenobiotic compounds in *in vitro* estuarine water and sediment / water system. *Chemosphere*, **17**, 2255-2271.
- Zhou, J. L., Fileman, T. W., Evans, S., Donkin, P. D., Mantoura, R. F., and Rowland, S., 1996 : Seasonal distribution of dissolved pesticides and polynuclear aromatic hydrocarbons in the Humber Estuary and Humber coastal zone. *Mar. Pollut. Bull.*, **32**, 599-608.

Studies on the regulatory mechanisms involved in the expression of heat-shock protein genes from rainbow trout^{*1}

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Abstract The present study was undertaken to examine the gene structure, the mRNA expression profiles and the mechanism of transcriptional regulation of fish heat-shock proteins (HSPs), using the rainbow trout *Oncorhynchus mykiss* as a model system.

Published studies using teleost cultured cells clearly demonstrate that teleosts can mount a stress response very similar to that observed with other vertebrates. However, HSP genes have only been cloned from a modest number of different fish species, and molecular studies of the fish HSPs are still in their early descriptive phase.

Heat-shock protein 70 (Hsp70) is the major stress-inducible protein in vertebrates and highly conserved throughout evolution. Rainbow trout have evolved by tetraploidization from a diploid ancestor, raising the possibility that multiple *Hsp70*s exist in the genome. To obtain full-length cDNA clones encoding Hsp70, a plasmid cDNA library was constructed from a heat-shocked juvenile of rainbow trout and screened. Consequently, two *Hsp70*s, *Hsp70a* and *Hsp70b*, were identified and found to have 98.1% identity in their deduced amino acid sequences. Southern blot analysis indicated that the two Hsp70s are encoded by distinct genes in the genome. Northern blot analysis showed that each of *Hsp70a* and *Hsp70b* expressed two mRNA species having different sizes by heat stress in rainbow trout RTG-2 cells.

To comprehensively analyze the mRNA expression profile of HSPs, multiple HSPs were isolated from RTG-2 cells and quantitatively compared for their mRNA levels between unstressed and heat-shocked cells using real-time quantitative RT-PCR analysis. To clone multiple HSP genes at once, 200 cDNAs were arbitrarily isolated from the cDNA library constructed from heat-shocked RTG-2 cells and sequenced. Consequently, nine cDNAs encoding HSPs were identified, namely, *Hsp90 β a*, *Hsp90 β b*, *Grp78*, *Hsp70a*, *Hsc70a*, *Hsc70b*, *Cct8*, *Hsp47*, and *DnaJ* homolog. By Northern blot analysis, single bands were detected for all genes examined except *Hsp70*s where two mRNA species having different sizes were detected in heat-shocked cells irrespective of *Hsp70a* and *Hsp70b*. Quantitative RT-PCR analysis showed that the accumulation levels of Hsp70a and Hsp70b mRNAs were dramatically increased after heat shock, and the mRNA levels in heat-shocked (28°C, 3 h) cells were 480- and 510-fold of those in controls, respectively. The increased levels of Hsc70a, Hsc70b and Hsp47 mRNAs were 1.3-, 2.8- and 1.6-fold, respectively, after heat shock. There were no differences in mRNA levels of the other four HSP genes between control and heat-shocked cells with a significance level of 0.05.

To demonstrate the existence of the heat-shock transcription factor (HSF) in rainbow trout, genes encoding HSF1 were cloned from RTG-2 cells. Consequently, two distinct *HSF1*s, named *HSF1a* and *HSF1b*, were identified. Southern blot analysis showed that each HSF1 is encoded by a distinct gene. The two HSF1 mRNAs were coexpressed in unstressed RTG-2 cells and in various tissues. In electrophoretic mobility shift assay, each *in vitro* trans-

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lated HSF1 bound to a cis-acting DNA element, the heat-shock element. Chemical cross-linking and immunoprecipitation analysis showed that HSF1a and HSF1b form heterotrimers as well as homotrimers.

The present study demonstrated that, in rainbow trout, at least some *HSP* and *HSF* have duplicate genes attributed to ancestral tetraploidization. This finding suggests that the comprehensive identification of duplicate genes is prerequisite to accurately examining gene expression profiles of rainbow trout cells. Since the mRNA expression profiles of HSPs may differ among stressors, it is interesting to compare the differences of the profiles for utilizing *HSPs* as a biomarker of various environmental stresses. Further, the present study suggests that a unique molecular mechanism, which functions through two distinct HSF1 isoforms, underlies the stress response in tetraploid and/or cold-water fish species such as rainbow trout. Since the lower activation temperature of rainbow trout HSF1 is a unique feature among vertebrate counterparts, a detailed comparison of rainbow trout and other vertebrate HSF1s will lead to further insight into the activation mechanism of the transcription factor.

Key words: gene expression profiling, gene expression regulation, heat-shock proteins, heat-shock response, *Oncorhynchus mykiss*.

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Section 1. Cloning of HSP cDNAs expressed in heat-shocked RTG-2 cells	ANOVA, analysis of variance
Section 2. Accumulation of HSP mRNAs in unstressed and heat-shocked RTG-2 cells	CCT, chaperonin containing TCP1
Section 3. Discussion	cDNA, complementary DNA
Chapter III. Cloning and characterization of HSF1	DBD, DNA-binding domain
Section 1. cDNA cloning and genomic organization of HSF1	DDBJ, DNA Data Bank of Japan
Section 2. mRNA expression pattern of HSF1 in various tissues	DIG, digoxigenin
	DNA, deoxyribonucleic acid
	EDTA, ethylenediaminetetraacetic acid
	EGS, ethylene glycol bis (succinimidylsuccinate)
	EMSA, electrophoretic mobility shift assay
	ER, endoplasmic reticulum
	EST, expressed sequence tag
	GRP, glucose-regulated protein
	HA, hemagglutinin
	HEPES, <i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -2-ethanesulfonic acid
	HR, hydrophobic repeat

HSC, heat-shock cognate
HSE, heat-shock element
HSF, heat-shock transcription factor
HSP, heat-shock protein
Ig, immunoglobulin
IOD, integrated optical density
mRNA, messenger RNA
NCBI, National Center for Biotechnology Information
NLS, nuclear localization signal
ORF, open reading frame
PCR, polymerase chain reaction
PVDF, poly(vinylidene difluoride)
RACE, rapid amplification of cDNA ends
RNA, ribonucleic acid
RT-PCR, reverse transcription polymerase chain reaction
SD, standard deviation
SDS, sodium dodecyl sulfate
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC, standard saline citrate
TCPI, t-complex polypeptide 1
TPR, tetratricopeptide repeat
UTR, untranslated region

Introduction

Poikilotherms like fish are directly affected by ambient temperature, which is among the most pervasive of environmental factors (Hawkins, 1996). Temperature influences their metabolism, activity levels, spawning, development and growth; and because of selective pressures associated with these processes, temperature is an ecological resource, influencing the proportion of potential habitat that is suitable for a species (Hawkins, 1996). In this manner, adaptive responses to ambient water temperature are essential for the survival of aquatic ectotherms. In particular, adaptation to heat stress is a critical factor for the viability of cold-adapted fish such as salmonids because they receive heat stress at moderately low temperatures. For instance, the ultimate incipient lethal temperature for rainbow trout, at which half of the fish tested die within 1 week, is about 26.2°C (Kaya, 1978).

At a cellular level, all organisms respond to heat stress by inducing the synthesis of a group

of proteins called the heat-shock proteins (HSPs) (Lindquist and Craig, 1988). The response is the most highly conserved genetic system known, existing in every organism in which it has been sought, from archaeobacteria to eubacteria, from plants to animals (Lindquist and Craig, 1988). HSPs are traditionally classified by molecular weights into several families (Sonna *et al.*, 2002). For example, a 70-kDa heat-shock protein belongs to the HSP70 family. HSP family members possess three principal biochemical functions: molecular chaperone activity, regulation of cellular redox state, and regulation of protein turnover (Sonna *et al.*, 2002).

The inducible HSP expression is regulated by the heat-shock transcription factors (HSFs) (Pirkkala *et al.*, 2001). In response to various inducers such as heat stress, most HSFs acquire DNA binding activity to the heat-shock element (HSE), thereby mediating transcription of the heat-shock genes, which results in accumulation of HSPs (Pirkkala *et al.*, 2001) as shown in Fig. 1. In vertebrates, several members of the HSF family, HSF1–4, have been found (Pirkkala *et al.*, 2001). Among the family members, HSF1 is functionally analogous to yeast and *Drosophila* HSF as the principal stress-induced transcription factor (Morimoto, 1998).

Published studies using teleost cultured cells clearly demonstrate that teleosts can mount a stress response very similar to that observed with other vertebrates (Hightower and Renfro, 1988). In regard to HSPs, most studies in fish have been performed at the protein level, and these results suggest that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish (Basu *et al.*, 2002). However, HSP genes have only been cloned from a modest number of different fish species, and molecular studies of the fish HSPs are still in their early descriptive phase (Basu *et al.*, 2002).

Thus, the present study was undertaken to examine the gene structure, the mRNA expression profiles and the mechanism of transcriptional regulation of fish HSPs. For this purpose, rainbow trout was chosen as an experimental model fish because of the following reasons. First, temperature tolerance is an important trait from both an economic and an evolutionary perspective in fishes,

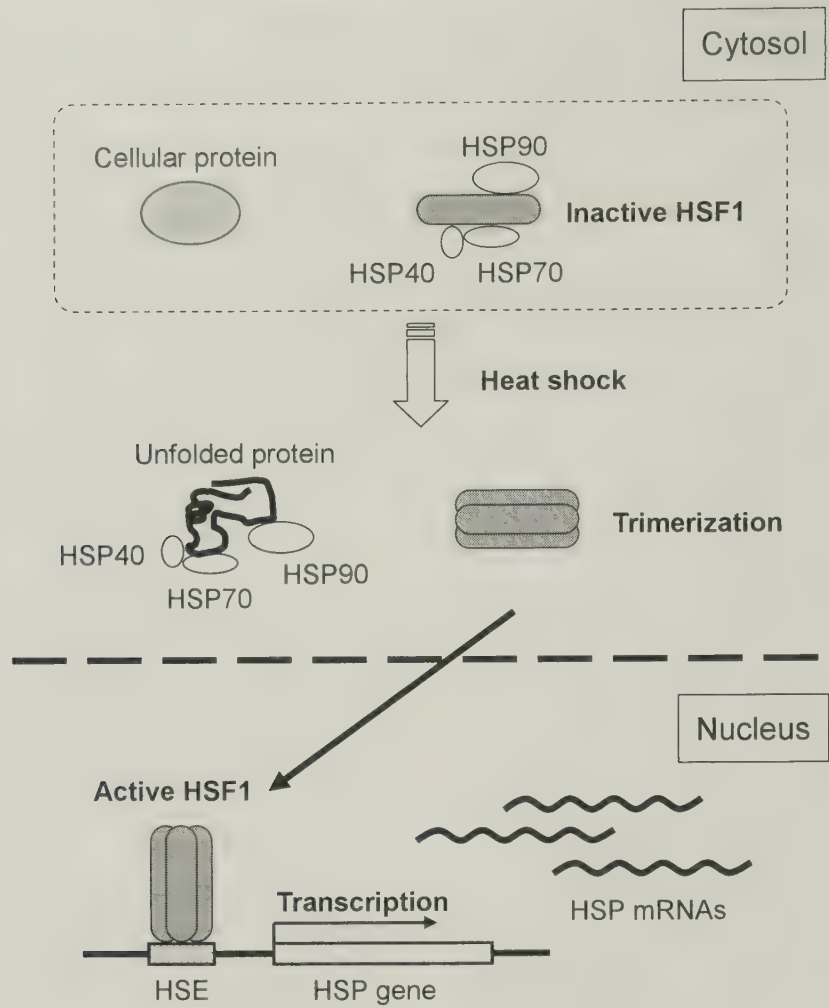


Fig. 1. Model of the regulatory mechanisms underlying the expression of heat-shock protein genes in mammalian cells. HSF1 is present in the cytosol as inactive monomers under normal conditions. The inactivation of HSF1 is associated with the binding of HSPs to the protein. When unfolded proteins accumulate in the cytosol by heat shock, HSPs dissociate from HSF1 to function as molecular chaperones. Subsequently, HSF1 acquires DNA binding ability through trimerization and translocates to the nucleus. HSF1 binds to HSEs in the upstream region of HSP genes and activates their transcription.

particularly among cold-water salmonids such as rainbow trout (Somorjai *et al.*, 2003). Second, since rainbow trout is one of the most intensively studied fishes in a wide range of research areas (Thorgaard *et al.*, 2002), considerable information is available about the physiology and biology of the fish as a background for molecular studies. Third, since rainbow trout have evolved by tetraploidization from a diploid ancestor (Ohno *et al.*, 1968), wider-scale DNA sequence information for the fish will provide an excellent and distinctive system for studying the aftermath of a genome-wide duplication event

and the associated structural and regulatory gene changes (Thorgaard *et al.*, 2002).

In rainbow trout, the major stress-inducible protein in the cells has been reported to be Hsp70 (Kothary and Candido, 1982), and partial cDNAs encoding Hsp70 have been isolated (Kothary *et al.*, 1984b). Based on these data, the present study began with isolation of a full-length cDNA encoding rainbow trout Hsp70. Chapter I describes the isolation of *Hsp70* and its expression profile in rainbow trout RTG-2 cells exposed to heat stress. Next, to comprehensively explore the expression

profile of HSP genes, isolation of genes encoding the other HSP family members was undertaken. Chapter II describes the isolation of HSP genes and their expression profiles in RTG-2 cells in response to heat stress. Finally, to investigate the molecular mechanism of transcriptional regulation of the HSP genes, isolation of a gene encoding HSF was undertaken. Chapter III describes the isolation and mRNA expression of *HSF1* and the activation of the transcription factor by heat stress.

According to the nomenclature used in the previous publication (Gething, 1997a), fully capitalized names were used to denote the protein family (e.g. HSP90, HSP70, HSP60 etc.) and initial capital letter was used for specific family members (e.g. Hsp90, Hsc70, Cct8 etc.) in this thesis.

Chapter I. Cloning and mRNA expression profile of Hsp70

Hsp70 is the major stress-inducible protein in vertebrates and highly conserved throughout evolution. The transcripts of Hsp70 are induced under various physiological stresses and the resulting translation products act as the molecular chaperone that mediates the correct assembly and localization of intracellular proteins (Miao *et al.*, 1997). In fish, *Hsp70s* have been cloned from a number of species; however, most studies of Hsp70 have been performed at the protein level (Basu *et al.*, 2002). Hence, relatively little is known about the expression profiles of Hsp70 transcripts in fish cells exposed to a variety of stresses.

Until now, two partial cDNA sequences encoding Hsp70 have been isolated from rainbow trout (Kothary *et al.*, 1984b). Their sequences have been used as probes to detect Hsp70 mRNA in rainbow trout cells exposed to stresses such as heat shock (Currie *et al.*, 1999; Kothary *et al.*, 1984a; Lund *et al.*, 2000; Samples *et al.*, 1999), sodium arsenite (Kothary *et al.*, 1984a), and hypoxia (Currie *et al.*, 1999). However, rainbow trout have evolved by tetraploidization from a diploid ancestor (Ohno *et al.*, 1968), raising the possibility that multiple *Hsp70s* exist in the genome, as inferred from Southern blot analysis (Kothary *et al.*, 1984b). Furthermore, a heat-shock cognate 70 (Hsc70) gene

has been identified in rainbow trout (Zafarullah *et al.*, 1992). Hsc70 is a member of the HSP70 family that is constitutively expressed in unstressed cells (Hightower and Leung, 1997). Nevertheless, only the partial, not full-length, cDNA sequences mentioned above have been used to detect rainbow trout Hsp70 transcripts. Therefore, an unambiguous interpretation on mRNA expression profiles of *Hsp70* and following evaluation of heat stress on rainbow trout were likely impossible. To this end, much more detailed information is required on the possible existence of the multiple *Hsp70s* and their sequence variations along with differences from *Hsc70*. The resulting information is prerequisite to utilization of *Hsp70* expression profiles as a molecular biomarker for stressed state of fish. Furthermore, it is interesting to explore different functional properties of the possible multiple Hsp70s of fish in terms of evolutionary interpretation.

Thus, the purpose of the study was to isolate full-length cDNA clones encoding rainbow trout Hsp70 and to investigate their mRNA expression profiles during heat stress. This chapter describes the identification of two distinct heat-inducible *Hsp70s* in rainbow trout and their different mRNA accumulation levels in cultured cells exposed to heat stress.

Section 1. Isolation and sequence analysis of cDNAs encoding HSP70

Rainbow trout is likely to have duplicate *Hsp70s* attributed to ancestral tetraploidization. Furthermore, since Hsp70 is a member of the highly conserved HSP70 family, homologous genes such as *Hsc70* are generally present in vertebrate genomes. Thus, to specifically analyze the mRNA expression profile of rainbow trout *Hsp70*, it is essential to identify the number of *Hsp70s* in rainbow trout genome and to determine their full-length DNA sequences. This section describes the isolation of full-length cDNAs encoding Hsp70 and their gene structures.

Materials and Methods

Construction of cDNA library

Rainbow trout *Oncorhynchus mykiss* were

obtained from the Nikko Station of the National Research Institute of Fisheries Science (Tochigi Prefecture, Japan) and reared on a commercial diet at 10°C. One individual of rainbow trout (3 months old) was heat-shocked for 30 min in an aquarium at 25°C, and the whole body was homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) using a power homogenizer (Polytron; Kinematica, Lucerne, Switzerland). The total RNA was isolated according to the manufacturer's instructions. Poly (A) RNA was purified from the total RNA using an Oligotex-MAG mRNA Purification kit (Takara Bio, Otsu, Japan). A directional cDNA library was constructed from poly(A) RNA using a SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Invitrogen) according to the manufacturer's instructions.

cDNA cloning

Screening of the cDNA library was performed using a GENETRAPPER cDNA Positive Selection System (Invitrogen). An oligonucleotide probe for a cDNA capture hybridization was designed based on the sequence of a rainbow trout *Hsp70* genomic clone isolated in a preliminary study (GenBank accession no. AB062281). The nucleotide sequence of the probe was 5'-TGGTCCTGGTGAA GATGAGG-3'. Biotinylation of the probe and cDNA capture hybridization were performed according to the manufacturer's instructions. The captured single-stranded DNA was repaired and transfected into ELECTROMAX DH10B cells (Invitrogen) by using an electroporation apparatus (MicroPulser; Bio-Rad Laboratories, Hercules, CA, USA). The electroporation was carried out at 1.8 kV using 20- μ l cells in a 0.1-cm-gap cuvette. White colonies were selected after transformation, and whether they were truly positive were analyzed by PCR using the above-mentioned probe sequence as a sense primer and a SP6 promoter primer (5'-GTG AACTATAGAAGAGCTATGACGTC-3') as an antisense primer. Subsequently, the positive clones were sequenced with an automated DNA sequencer (Gene Rapid; Amersham Biosciences, Piscataway, NJ, USA) after labeling with a Thermo Sequenase Cy5.5 Dye Terminator Sequencing kit (Amersham Biosciences).

Phylogenetic analysis

Amino acid sequences of vertebrate *Hsp70*s and *Hsc70*s were retrieved from the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp/Welcome-e.html>). A phylogenetic tree was constructed from the multiple sequence alignment by using the neighbor-joining method (Saitou and Nei, 1987), as implemented in CLUSTAL W (Thompson *et al.*, 1994). A graphical output of the tree was visualized by the program TreeView (Page, 1996).

Southern blot analysis

Genomic DNA was isolated from RTG-2 cells by using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each 10- μ g DNA was digested with restriction enzymes *Bam*HI, *Eco*RI, and *Hind*III, electrophoresed on a 1% agarose gel, and capillary-transferred to a nylon membrane (Hybond N+; Amersham Biosciences) with 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA was UV-crosslinked to the membranes, which were subsequently hybridized for 1 h in PerfectHyb hybridization solution (Toyobo, Osaka, Japan) with digoxigenin (DIG)-labeled DNA probes. Probes specific for *Hsp70a* and *Hsp70b* were designed in their 3'-untranslated regions (UTRs), namely, nucleotides 2561-2773 and 2029-2191, respectively. The GenBank accession numbers of the two *Hsp70*s are as follows: *Hsp70a*, AB176854 and *Hsp70b*, AB176855. The probes were labeled with a PCR DIG Probes Synthesis kit (Roche Diagnostics, Mannheim, Germany). The hybridized membranes were washed twice with 2 $\times$ SSC plus 0.1% SDS at room temperature for 5 min, and then twice with 2 $\times$ SSC plus 0.1% SDS at 68°C for 15 min. The chemiluminescent detection of the probes was performed with a DIG Luminescent Detection kit (Roche Diagnostics) according to the manufacturer's instructions. The positive signals were detected by exposure on Hyperfilm-ECL (Amersham Biosciences).

Results

To obtain full-length cDNA clones encoding *Hsp70*, a plasmid cDNA library containing 1.1 $\times$

10⁶ independent clones was constructed from a heat-shocked juvenile of rainbow trout and screened. After cDNA capture hybridization and *Escherichia coli* transformation, 14 out of 93 colonies were PCR-positive. Each positive clone was partially sequenced (approximately 400 bases) from the 5' end and, consequently, 12 out of 14 clones showed sequences highly homologous to those of Hsp70 cDNAs previously reported for other eukaryotic species. Moreover, the clones were divided into two groups based on the nucleotide sequences, and each group was comprised of six identical clones. The clones having the longest size from each group were selected, named C1 and C11, and their complete cDNA sequences were determined. Consequently, clones C1 and C11 were 2804 and 2229 bp in length, respectively. Hereafter, these are referred to as *Hsp70a* and *Hsp70b*, respectively.

The deduced amino acid sequences of *Hsp70a* and *Hsp70b* both contain 644 residues with calculated molecular weights of 71 015 and 70 988, respectively (Fig. 2). The two proteins shared 98.1% identity with variations in 12 amino acid residues. Furthermore, rainbow trout Hsp70s shared many identical amino acid residues with those of other vertebrates (Fig. 2). For example, Hsp70a and Hsp70b showed 84.5% and 84.2% identity with human Hsp70.1, respectively.

Fig. 3 shows a phylogenetic tree constructed with cytosolic HSP70 family members in vertebrates. The tree was composed of the three major groups: mammalian Hsp70, fish Hsp70, and vertebrate Hsc70. Among the three groups in the tree, rainbow trout Hsp70s belonged to the fish Hsp70 group, confirming the isolated cDNA clones to be those of *Hsp70s*.

Fig. 4 shows the Southern blots of rainbow trout *Hsp70a* and *Hsp70b*. To accurately distinguish between the two genes, the DNA probes were designed to hybridize to 3'-UTR specific to respective genes. Consequently, the band patterns differed between *Hsp70a* and *Hsp70b*, suggesting that the two rainbow trout Hsp70s were encoded by distinct genes in the genome.

Section 2. mRNA expression profiling of *Hsp70*—Temperature shift experiment

In the preceding section, two distinct genes

encoding Hsp70 were identified in rainbow trout, and the nucleotide sequences of their 3'-UTR were found to have low homology with each other. Indeed, each *Hsp70* was specifically detected in Southern blot analysis by using the part of their 3'-UTR sequences as DNA probes. By applying the same probes to Northern blot analysis, this section describes the mRNA expression profiling of *Hsp70s* in RTG-2 cells exposed to different temperatures.

Materials and Methods

Cell cultures and exposure to heat stress

RTG-2 cells, immortalized rainbow trout gonadal fibroblasts (Wolf and Quimby, 1962), were cultured at 20°C in Leibovitz's L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum and antibiotic-antimycotic mixture (100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Invitrogen). The culture dishes were sealed with Parafilm and immersed into a water bath at 24, 28, and 32°C for 1 h each.

Northern blot analysis

Total RNA was isolated from RTG-2 cells with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For Northern blotting, 5 µg of the total RNA was separated on a 1.2% agarose-formaldehyde gel and capillary transferred to a Hybond N+ nylon membrane with 10 × SSC. Hybridization and detection of target genes were performed through Southern blot analysis as described in Section 1 of this chapter. A probe specific for *Hsc70* was designed in its 3'-UTR, namely, nucleotides 6188–6408, based on the genomic DNA sequence (GenBank accession no. S85730) previously reported (Zafarullah *et al.*, 1992).

Quantification and statistical analysis of mRNA accumulation levels

The fluorograms of Northern blots were scanned in 8-bit grayscale into Adobe Photoshop (Adobe Systems, San Jose, CA, USA) at 300 dpi using a flatbed scanner (GT-8700F; Seiko Epson, Tokyo, Japan) and accompanying software. The integrated optical density (IOD) of positive bands was quantified using Gel-Pro Analyzer software

1	MSSAKGPSIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFDTERLIGDAAKNQVAMNPNTVF	rainbow trout Hsp70a
1		rainbow trout Hsp70b
1	...P...IA.....S.....NA.....L.....	zebrafish Hsp70
1	--...KTA.....L...Q...	rat Hsp70.1/2
1	--...AAA.....L...Q...	human Hsp70.1
71	DAKRLIGRKFNQVQADMKHWPFKVVS DGGKPKVQVDYKGENKSFNPEEISSMVLVKMREIAEAYLGQK	rainbow trout Hsp70a
71	C.....	rainbow trout Hsp70b
71	R.D.H...S...S...R.....A.EHS..D.T.....K.....	zebrafish Hsp70
69	G.P...S.....Q..N..D.....N.....R..Y.....T..K.....HP	rat Hsp70.1/2
69	G.P...S.....Q.IN..D.....S...T.A.Y.....T..K.....YP	human Hsp70.1
141	VNSAVITVPAYFNDSQRQATKDAGVIAGLNVLRINEPTAAAIAYGMDKGKSRERNVLIFDLGGGTFDVS	rainbow trout Hsp70a
141	M.....	rainbow trout Hsp70b
141	.T.....E.H.....V.....A.....RP.....L.....S.....E.....	zebrafish Hsp70
139	.T.....L.RTGKG.....	rat Hsp70.1/2
139	.T.....L.RTGKG.....	human Hsp70.1
211	ILTIEDGIFEVKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSS	rainbow trout Hsp70a
211	I.....	rainbow trout Hsp70b
211	V.....T.N.....	zebrafish Hsp70
209	...D.....D.....V.....T	rat Hsp70.1/2
209	...D.....N.....V.....T	human Hsp70.1
281	QASIEIDSLFEGIDFYTSITRARFEEMCSDLFRGTLEPVEKALRDAKMDKAQIHDVVVLVGGSTRIPKVQK	rainbow trout Hsp70a
281	R.....G.....	rainbow trout Hsp70b
281	Y.....L.....D.....I.....I..	zebrafish Hsp70
279	..L.....L.....L.....L.....L.....	rat Hsp70.1/2
279	..L.....L.....S.....L.....L.....	human Hsp70.1
351	LLQDFNFNGRELNKSINPDEAVAYGAAIQAAILSGDKSENVQDLLLLDVAPLSLGIETAGGVMTALIKRNT	rainbow trout Hsp70a
351	V.....M..T.G...M.....	rainbow trout Hsp70b
349	D.....V.....M.....L.....S	zebrafish Hsp70
349	D.....V.....M.....L.....S	rat Hsp70.1/2
349	D.....V.....M.....L.....S	human Hsp70.1
421	TIPSKQTQIFTTYSNQPVGMIQVYEGERAMTKDNLLGKFELSGIPSPRGVPQIEVTFDIDANGILNV	rainbow trout Hsp70a
421	T.....A.....	rainbow trout Hsp70b
421	...T...T.A.....L...F...G.....D.T...A.....	zebrafish Hsp70
419	...T...T.....L.....R.....R.....A.....	rat Hsp70.1/2
419	...T...T.....L.....R.....A.....	human Hsp70.1
491	AAVDKSTGKENKITITNDKGRLSKEDIERMVQDADKYKAEDDAQREKMAAKNSLESYAFNMKSSVEDDNM	rainbow trout Hsp70a
491	S.....I.....	rainbow trout Hsp70b
491	S.A.....Q.....E.....E.....L...IS.....N.....L	zebrafish Hsp70
489	T.T.....A.....E.....E.ER.....EV...RV...A.....A...EGL	rat Hsp70.1/2
489	T.T.....A.....E.....E.E.....EV...RVS...A.....A...EGL	human Hsp70.1
561	KGKISEEDKKKVMDCRNTISWLENNQLGDKEEYEHQLKELEKVCQPIITKLYQQGGMPTGCCGDQARTS	rainbow trout Hsp70a
561	Q.....V...D.....	rainbow trout Hsp70b
561	R.VEK..EAV.R.....A.....N.V.S...-...A.G..A...AA	zebrafish Hsp70
559	A.....L.K.QEV...DS.T.AE...FV.KRE...R..N...SG...GA.A.-.AG.FG.QAP	rat Hsp70.1/2
559	A.....L.K.QEV...DA.T.AE.D.F..KR...Q..N...SG...GA.G.-.PG.FG.QGP	human Hsp70.1
631	SGVSSQGPTIEEID	rainbow trout Hsp70a
631	..D.....	rainbow trout Hsp70b
630	..A.A.....V.	zebrafish Hsp70
628	K.G.GS.....V.	rat Hsp70.1/2
628	K.G.GS.....V.	human Hsp70.1

Fig. 2. Comparison of the amino acid sequences of rainbow trout Hsp70a and Hsp70b with those of other vertebrate Hsp70s. Dots represent amino acid residues that are identical to those of the rainbow trout Hsp70a sequence, and dashes indicate gaps set to maximize the alignment. The box represents the location of the sequence used for designing a probe to screen rainbow trout *Hsp70*s. GenBank accession numbers for cited genes are presented in Fig. 3.

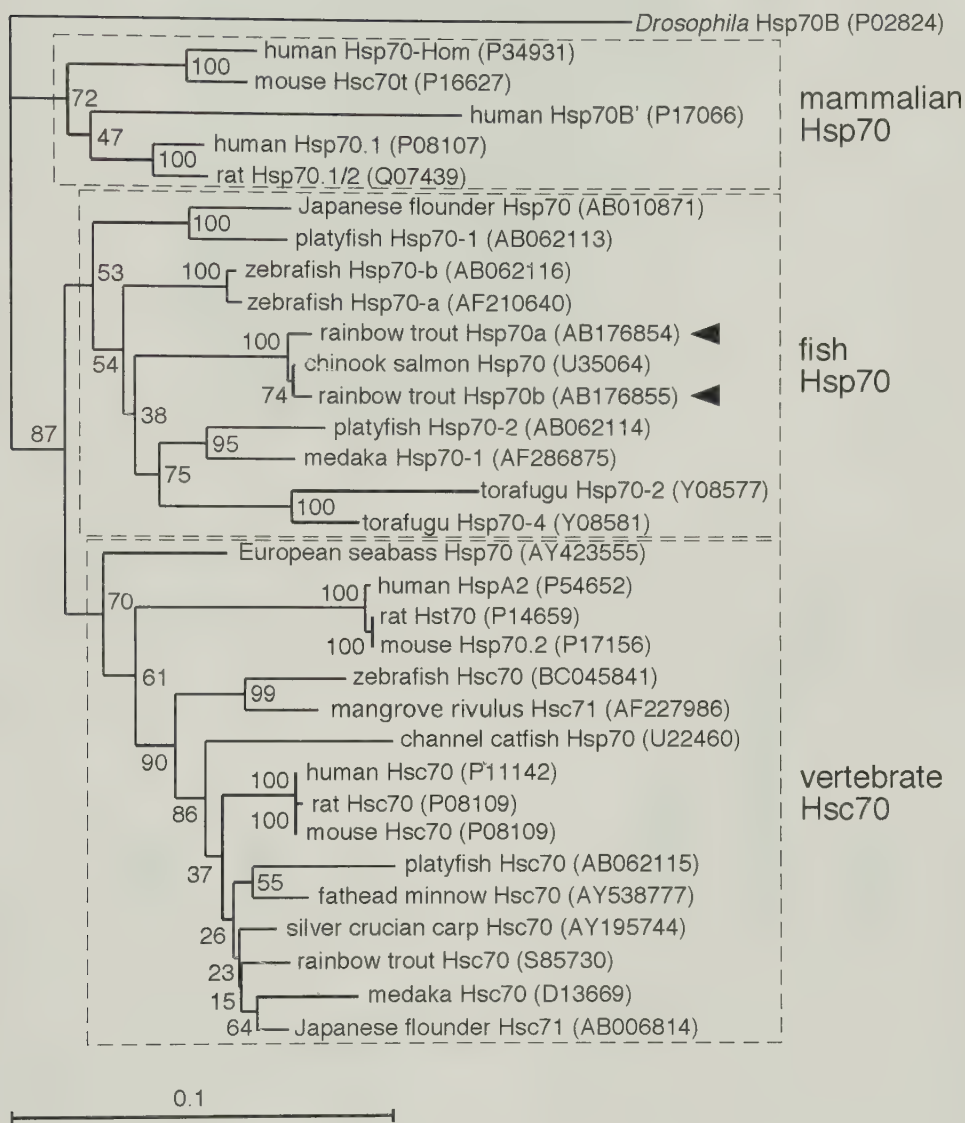


Fig. 3. Phylogenetic analysis of cytosolic HSP70 family members in vertebrates. Arrowheads indicate the position of rainbow trout Hsp70a and Hsp70b. The three groups of HSP70 family members are boxed. *Drosophila* Hsp70B was used as an outgroup. Numbers at the nodes represent the percentage of bootstrap values (1000 replicates). The scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank or Swiss-Prot accession numbers for cited proteins are indicated in parentheses.

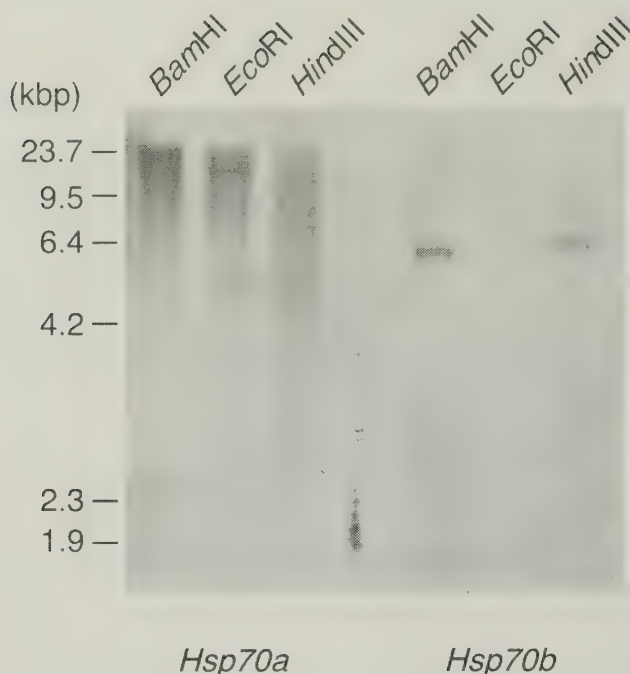


Fig. 4. Genomic Southern blot analysis of rainbow trout *Hsp70a* (left panel) and *Hsp70b* (right panel). RTG-2 genomic DNA digested with *Bam*HI, *Eco*RI, and *Hind*III was separated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with DIG-labeled probes. The probes are complementary to sequences in 3' -UTR of the two genes. λ DNAs digested with *Hind*III were used as size markers and are indicated on the left.

(Media Cybernetics, Silver Spring, MD, USA). The relative amounts of *Hsp70* mRNAs were calculated by dividing IOD of *Hsp70* by that of *Hsc70*. To confirm the validity of using *Hsc70* as an internal standard, possible changes in the amounts of *Hsc70* mRNAs themselves during heat stress were tested with one-way analysis of variance (ANOVA) at the significance level of $P < 0.05$.

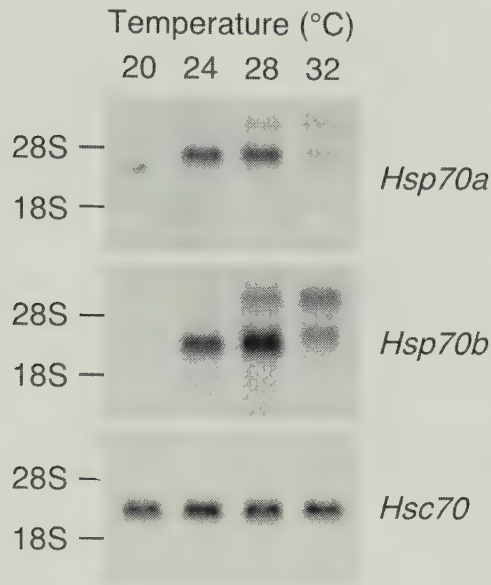
Results

Fig. 5 shows the expression profiles of rainbow trout *Hsp70* mRNAs in RTG-2 cells exposed to different heat shock temperatures for 1 h. Neither *Hsp70a* nor *Hsp70b* mRNAs were detected at the control temperature (20°C), whereas both mRNAs were apparently induced at 24°C and above (Fig. 5A). In contrast, *Hsc70* mRNA was constitutively expressed at all temperatures examined (Fig.

5A). A single mRNA species was detected at 24°C for both *Hsp70*s, where the length of *Hsp70a* mRNA differed from that of *Hsp70b* mRNA. The apparent sizes of the bands were consistent with the calculated ones based on the cloned cDNAs described in the preceding section. Besides the single mRNA species at 24°C, longer mRNA species were detected at 28 and 32°C for each *Hsp70* (Fig. 5A). Hereafter, for convenience, the shorter and longer mRNA species are referred to as *Hsp70*-short and *Hsp70*-long mRNAs, respectively.

To compare the expression levels of different mRNA species, the amounts of the *Hsp70* transcripts were determined by measuring IOD of the bands. IOD of *Hsc70* mRNA was used as an internal standard because there was no statistically significant difference in the level of *Hsc70* mRNA at different temperatures ($P > 0.05$, one-way ANOVA). In this regard, however, IOD at 32°C was excluded

A



B

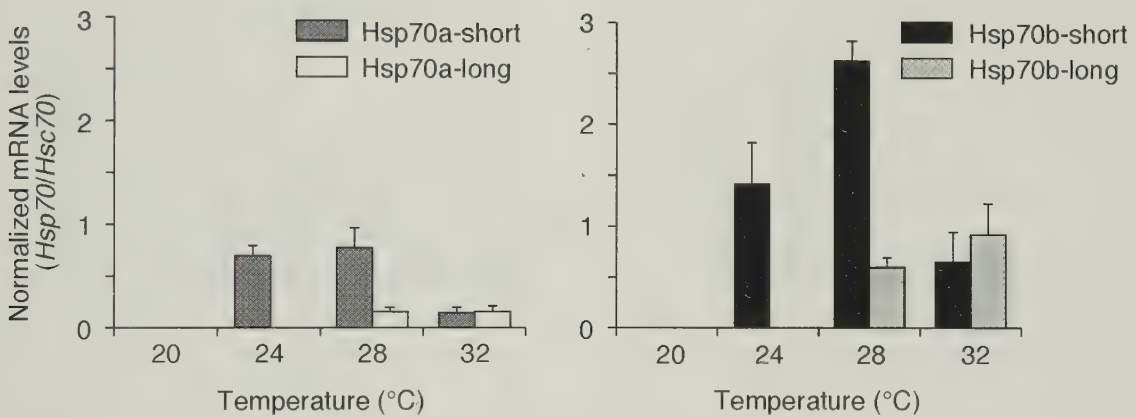


Fig. 5. Expression profiles of Hsp70 mRNAs in RTG-2 cells exposed to different temperatures. (A) Northern blot analysis of *Hsp70a*, *Hsp70b*, and *Hsc70*. The probes are complementary to sequences in 3' -UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments. (B) Quantification of Northern blot analysis. The amounts of Hsp70 mRNAs normalized to those of *Hsc70* mRNAs are shown. The values were calculated by dividing IOD of Hsp70 mRNA by that of *Hsc70* mRNA, and are presented as means $\pm$ SD of three independent experiments.

from the ANOVA because of its large variance. Therefore, the normalized mRNA levels of Hsp70 at 32°C were calculated for reference. As shown in Fig. 5B, Hsp70b-short and Hsp70b-long mRNAs displayed higher expression levels than Hsp70a counterparts at any heat shock temperatures tested. For example, the mean value of Hsp70b-short mRNA was 3.4-fold higher than that of Hsp70a-short mRNA at 28°C. In addition, the expression level of Hsp70a-short mRNA was almost as high as that of Hsc70 mRNA at 24 and 28°C, whereas the maximum expression level of Hsp70b-short mRNA was 2.6-fold higher than that of Hsc70 (Fig. 5B).

Section 3. mRNA expression profiling of *Hsp70* — Time course experiment

In the preceding section, a 1 h heat shock at 28°C gave rise to the highest accumulation levels of Hsp70 mRNAs. When heat shock is prolonged, how do the mRNA levels change in RTG-2 cells? To answer this question, this section describes the mRNA expression profiling of *Hsp70*s during prolonged heat stress.

Materials and Methods

Cell cultures and exposure to heat stress

RTG-2 cells were cultured at 20°C as described in Section 2 of this chapter. The culture dishes were sealed with Parafilm and immersed into a water bath at 28°C for 1.5, 3, 6, 12, and 24 h.

Northern blot analysis

Northern blot analysis was performed as described in Section 2 of this chapter.

Quantification and statistical analysis of mRNA accumulation levels

Quantification and statistical analysis of mRNA expression levels were performed as described in Section 2 of this chapter.

Results

Fig. 6 shows the expression profiles of Hsp70 mRNAs during prolonged heat stress at 28°C. Since

there was no statistically significant changes in the level of Hsc70 mRNA during heat stress ($P>0.05$, one-way ANOVA), IOD of Hsc70 mRNA was again used as an internal standard. In this experiment, Hsp70 mRNA levels reached the maximum after 3 h at 28°C and then decreased (Fig. 6B). Hsp70a-long and Hsp70b-long mRNAs disappeared by 12 h, whereas Hsp70a-short and Hsp70b-short mRNAs remained detectable after 24 h. Although *Hsp70a* and *Hsp70b* had similar expression profiles, the levels of Hsp70b-short and Hsp70b-long mRNAs were consistently higher than Hsp70a counterparts during heat stress (Fig. 6B).

Section 4. Discussion

In this chapter, two distinct *Hsp70*s were identified in rainbow trout, and their mRNA expression profiles were separately investigated using RTG-2 cells exposed to heat stress.

Considering the possibility that multiple *Hsp70*s are expressed in rainbow trout cells, a screening to identify the cDNAs was carefully designed. First, to eliminate individual variations, the cDNA library was constructed using poly(A) RNA prepared from one individual of juvenile rainbow trout. Second, 12 full-length cDNA clones encoding Hsp70 were isolated and correctly classified by DNA sequencing. Consequently, it was found that two *Hsp70*s, *Hsp70a* and *Hsp70b*, were expressed in rainbow trout. Until now, two cDNA clones have been isolated as family members of *Hsp70*s from rainbow trout: *THS70.7* and *THS70.14* (Kothary *et al.*, 1984b); however, both clones were only partially sequenced. Comparing the partial cDNAs with the complete cDNA sequences isolated in the present study, *THS70.14* was found to correspond to *Hsp70a*. On the other hand, it could not be identified with certainty that *THS70.7* is either *Hsp70a* or *Hsp70b* because *THS70.7* was the fragment of the coding region of *Hsp70* that is highly conserved. In addition, a genomic clone of rainbow trout *Hsp70* was previously isolated (GenBank accession no. AB062281), which corresponds to *Hsp70a* and has an intronless open reading frame.

Rainbow trout *Hsp70*s were highly homologous to those from other vertebrates (Fig. 2). Phylogenetic

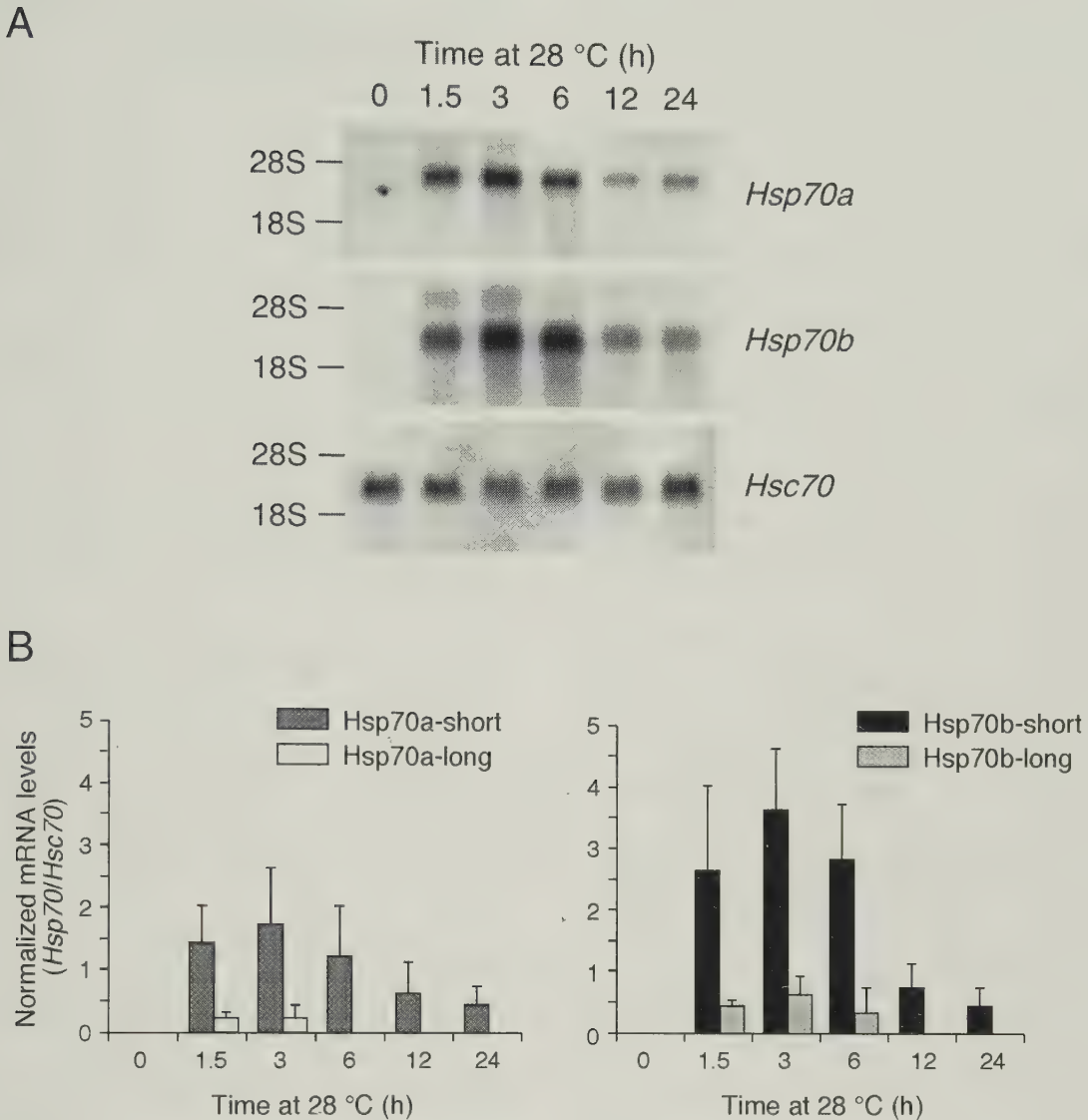


Fig. 6. Expression profiles of Hsp70 mRNAs in RTG-2 cells exposed to prolonged heat stress at 28°C. (A) Northern blot analysis of *Hsp70a*, *Hsp70b*, and *Hsc70*. The probes are complementary to sequences in 3'-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments. (B) Quantification of Northern blot analysis. The amounts of Hsp70 mRNAs normalized to those of *Hsc70* mRNAs are shown. The values were calculated by dividing IOD of Hsp70 mRNA by that of *Hsc70* mRNA, and are presented as means $\pm$ SD of three independent experiments.

analysis of cytosolic Hsp70 family members indicates that there is a fish Hsp70 group containing rainbow trout Hsp70s (Fig. 3). Moreover, the phylogenetic tree showed the other two groups: mammalian Hsp70 and vertebrate Hsc70. Even if other Hsp70 family members, glucose-regulated protein 75 (Grp75) and 78 (Grp78), were included in the analysis, the clustering of the three groups was not altered (data not shown). Some *Hsp70s* belonging to the fish Hsp70 group are heat-inducible; e.g., Japanese flounder *Paralichthys olivaceus Hsp70* (Yokoyama *et al.*, 1998), platyfish *Xiphophorus maculatus* and zebrafish *Danio rerio Hsp70s* (Yamashita *et al.*, 2004). Therefore, rainbow trout Hsp70s are suggested to be heat-inducible forms, and indeed, their transcription was induced by heat stress (Figs. 5 and 6).

Southern blot analysis indicated that rainbow trout Hsp70a and Hsp70b are encoded by distinct genes in the genome (Fig. 4), which would be common among salmonid fishes because, as suggested by Ohno *et al.* (1968), most of them have evolved by tetraploidization from a diploid ancestor. In fact, Rise *et al.* (2004) have reported the presence of distinct forms of several genes in Atlantic salmon *Salmo salar* by preliminary comparisons of its expressed sequence tag (EST) clusters. Thus, rainbow trout *Hsp70a* and *Hsp70b* are suggested to be paralogous genes.

Northern blot analysis showed that both *Hsp70a* and *Hsp70b* were induced by heat stress in RTG-2 cells (Figs. 5 and 6). In this analysis, Hsp70a and Hsp70b mRNAs could be clearly distinguished by using probes specific to respective genes. As shown in Fig. 5, the mRNA levels of *Hsp70a* and *Hsp70b* decreased at 32°C and the variance of data at this temperature was large, suggesting that a temperature of 32°C was too severe for rainbow trout cells. For instance, the synthesis of Hsps was not observed at 32°C in RTG-2 cells, and the temperature range in which RTG-2 cells could survive for at least 7 days was 0–28°C (Mosser *et al.*, 1986). Furthermore, Mosser *et al.* (1986) reported that RTG-2 cells became shriveled and took on a threadlike appearance after 1 h at 32°C. The observation in the present study was consistent with these results, and there was no observable change

in the cell morphology after 1 h at 24 and 28°C (data not shown). Thus, temperatures exceeding 28°C would be too stressful for RTG-2 cells.

A new finding is that longer mRNA species were induced in rainbow trout cells at above 28°C for both *Hsp70a* and *Hsp70b* (Fig. 5). Although the expression of multiple mRNA species has been reported for several heat-shock genes (Dellavalle *et al.*, 1994; Takechi *et al.*, 1994), two different mRNA species from a single *Hsp70* have not yet been detected. Since the longer mRNA species were detected only under severe heat stress conditions, they may be useful as a biomarker for the high degrees of heat stress. However, whether such mRNAs are induced at the individual level remains to be clarified. Now, what mechanisms underlie the generation of different Hsp70 mRNA species under severe heat stress condition? There are three possible mechanisms: (i) polyadenylation and deadenylation [e.g., *Drosophila Hsp70* (Dellavalle *et al.*, 1994)], (ii) defective splicing [e.g., *Drosophila Hsp83* (Dellavalle *et al.*, 1994)], and (iii) alternative splicing [e.g., mouse *Hsp47* (Takechi *et al.*, 1994)]. Among them, it is most likely that the mRNA species are generated by alternative splicing. One reason is that the lengths of the longer mRNA species appear to be too long if they are polyadenylated forms of the shorter mRNA species (Figs. 5A and 6A). For example, a polyadenylated form of *Drosophila Hsp70* mRNAs has poly(A) tails of about 120 to 160 nucleotides (Dellavalle *et al.*, 1994). Another reason is that rainbow trout Hsc70 mRNA, which contains introns in the genomic DNA (Zafarullah *et al.*, 1992), showed no change in length at above 28°C (Fig 5A). This indicates that splicing is not defective after severe heat stress. Taken together, the longer mRNA species of the rainbow trout *Hsp70s* are thought to be alternatively spliced products. However, the possibility that polyadenylation and deadenylation may be involved in the event cannot be excluded because the sequences of poly(A) tails from the longer mRNA species have not yet been determined.

Another finding is that the accumulated mRNA levels of *Hsp70b* were higher than that of *Hsp70a* during heat stress (Figs. 5 and 6). This difference seems not due to the difference of labeling

efficiency between the two DIG-labeled probes. The efficiency for both probes were evaluated by gel electrophoresis, confirming that there was no significant difference between the signal intensities of the two bands (data not shown). However, quantitative PCR analysis will be needed to discuss more accurately the difference of the mRNA levels. On the other hand, the mRNA expression profiles of the two genes were similar to each other in both temperature shift (Fig. 5) and time course experiments (Fig. 6). In the time course experiment, prolonged heat stress at 28°C for longer than 3 h caused a decrease in Hsp70 mRNA levels (Fig. 6B). These results generally agreed with the induction pattern of Hsp70 mRNA previously reported (Kothary *et al.*, 1984a), in which, however, multiple Hsp70 mRNA species were examined together without separation.

At the protein level, the synthesis of Hsp70 has been reported to attain a peak at 2 h and maintain high levels between 2 and 6 h after treatment of RTG-2 cells at 28°C (Mosser *et al.*, 1986). These results are roughly consistent with the mRNA expression profiles of Hsp70 shown in Fig. 6. In addition, Mosser *et al.* (1987) reported that the induction of HSP synthesis at 28°C paralleled the development of thermotolerance in RTG-2 cells. On the other hand, rainbow trout Hsp70a and Hsp70b have not been clearly distinguished at the protein level as well as at the mRNA level. Nevertheless, two proteins supposed to be Hsp70 have been detected in heat-stressed RTG-2 cells (Airaksinen *et al.*, 1998; Mosser *et al.*, 1986). Their apparent molecular sizes were 70 and 68 kDa (Mosser *et al.*, 1986), and 69 and 67 kDa (Airaksinen *et al.*, 1998). Considering the predicted molecular weights described in Results of Section 1, Hsp70a and Hsp70b seem to be their equivalents.

Chapter II. Cloning and mRNA expression profiles of HSP family members

To resolve the regulation and functional significance of HSPs, fish are ideal organisms because they are ectothermic vertebrates and naturally exposed to thermal stress in their environment (Basu *et al.*, 2002). However, most studies on HSPs in fish

have been performed at the protein level, and HSP genes have only been cloned from a modest number of different species (Basu *et al.*, 2002). Furthermore, since the structure and the mRNA expression of HSP genes have been analyzed individually and separately, a comprehensive mRNA expression profile of HSP genes has not been established in cells from a single fish species. Additionally, HSPs are good examples of molecular level biomarkers to perceive sublethal cellular damages as the result of an environmental stress (Ryan and Hightower, 1996). However, the use of HSPs as indicators for stress states of fish in general is premature (Iwama *et al.*, 2004), in part because little information is available about a comprehensive stress response profile of HSP genes in fish cells.

Thus, this chapter describes the isolation of multiple HSP genes from rainbow trout and analysis of their expression profiles at the mRNA levels. In this regard, the mRNA levels between unstressed and heat-shocked cells were quantitatively compared using real-time RT-PCR analysis.

Section 1. Cloning of HSP cDNAs expressed in heat-shocked RTG-2 cells

To comprehensively analyze the mRNA expression profile of HSPs, DNA sequence information for each gene is prerequisite to the specific detection. However, HSPs have not been cloned from rainbow trout except for *Hsc70* reported by Zafarullah *et al.* (1992) and *Hsp70s* described in the preceding chapter. Thus, isolation of genes encoding HSP family members is required, and it is desirable that HSPs would be isolated as many as possible at once. This section describes an efficient isolation of cDNAs encoding HSPs from RTG-2 cells and the features of their gene structures.

Materials and Methods

Cell Culture

RTG-2 cells were cultured at 15°C as described in Section 2 of Chapter I.

Construction of directional cDNA library

Five 100-mm culture dishes with confluent

RTG-2 cells were heat-shocked at 25°C for 24 h in an incubator. Cells were lysed with TRIzol Reagent (Invitrogen), and total RNA was isolated according to the manufacture's instructions. Poly (A) RNA was isolated from the total RNA using an Oligotex-MAG mRNA Purification kit (Takara Bio). A directional cDNA library was constructed using a SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Invitrogen). cDNAs were constructed from 5 µg of poly(A)

RNAs using a *NotI* primer-adapter. After addition of a *SalI* adapter, cDNAs were digested with *NotI* and size-fractionated by column chromatography according to the manufacturer's instructions. The digests were ligated into plasmid vector pSPORT1, and the plasmids were introduced into ELECTROMAX DH10B competent cells (Invitrogen) using an electroporation apparatus (MicroPulser; Bio-Rad Laboratories). The electroporation was performed using a 0.1-cm-gap cuvette containing 1 µl of the

Table 1. List of cDNAs isolated from heat-shocked RTG-2 cells

Putative genes	No. of clone	% of total clones
HSP family members		
heat-shock cognate protein 70	3	
heat-shock protein 90β	2	
heat-shock protein 70	2	
glucose-regulated protein 78	1	
chaperonin containing TCP1, subunit 8 (Cct8)	1	
heat-shock protein 47	1	
DnaJ (Hsp40) homolog, subfamily A, member 4	1	
Subtotal	11	5.5
Chaperone-related proteins		
progesterone receptor-related protein p23	2	
14-3-3B2 protein	1	
FK506 binding protein 2	1	
Subtotal	4	2.0
Others		
cytochrome <i>c</i> oxidase subunit I	6	
elongation factor 1α	5	
cytoskeletal β-actin	4	
ferritin H-2	4	
K18, simple type I keratin	4	
CD81	2	
cyclin G1	2	
sorting nexin	2	
thymosin β	2	
trafficking protein particle complex 5	2	
type I collagen α2 chain	2	
singletons	71	
Subtotal	106	53.0
Unknown	79	39.5
Total	200	100.0

Table 2. Summary of cloned cDNAs encoding HSP family members

HSP family	Rainbow trout RTG-2 cDNA			The best matching sequence in the blastp program			
	Clone no.	Designation	Accession no.	Protein name	Species	Accession no.	Amino acid identity (%)
HSP90	Q120	<i>Hsp90βa</i>	AB196457	Hsp90β	Atlantic salmon	AF135117	98.9
	R046	<i>Hsp90βb</i>	AB196458	Hsp90β	Atlantic salmon	AF135117	97.8
HSP70	R032 ^a	<i>Grp78</i>	AB196459	Grp78	zebrafish	BC052971	90.1 ^b
	R058	<i>Hsp70a</i>	AB176854 ^c	Hsp70	rainbow trout	AB062281 ^d	99.4
	Q126	<i>Hsc70a</i>	AB196460	Hsc71	rainbow trout	S85730 ^d	100.0
	Q110 ^a	<i>Hsc70b</i>	AB196461	Hsc71	rainbow trout	S85730 ^d	79.2 ^b
HSP60	Q123	<i>Cct8</i>	AB196462	Cct8	zebrafish	BC050492	90.7
HSP47	R100	<i>Hsp47</i>	AB196463	Hsp47	zebrafish	BC071301	83.5
HSP40	Q086 ^{a,e}	<i>DnaJ</i> homolog	AB196464	DnaJ homolog	<i>Xenopus</i>	BC042291	78.9 ^b

^a cDNA clone with an incomplete 5' end.
^b partial sequence identity.
^c cDNA clone isolated in Chapter I.
^d genomic clone.
^e cDNA clone containing putative intron(s).

plasmid DNA and 20 μl of the competent cells at 1.8 kV and one pulse. The cells were plated onto LB agar plates containing 100- μg/ml ampicillin.

Sequencing and bioinformatic analysis

Two hundred white colonies were arbitrarily selected for isolation of plasmid DNA. The plasmid DNA was purified using a conventional alkaline/SDS lysis method. The 5' end each of cDNA inserts was sequenced with a T7 promoter primer (5' -TAATACGACTCACTATAGG-3') using a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Biosciences) and an automated DNA sequencer (373A; Applied Biosystems, Foster City, CA, USA). After removing the vector sequence, the deduced amino acid sequences were compared with sequences in the nr protein database using the blastx program in the NCBI BLAST homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Results

To clone multiple HSP genes at once, 200 cDNAs were arbitrarily isolated from heat-shocked RTG-2 cells and their 5' ends were sequenced. The readable sequence length was approximately 340 bp. The data

are summarized in Table 1, where the BLAST hits with an *E* value≥10⁻⁵ were defined as unknown. Out of 200 clones, 121 clones (60.5%) showed significant similarities (*E* value<10⁻⁵) with proteins in the NCBI database, and 11 clones (5.5%) were putatively identified as HSP family members. Additionally, four clones (2.0%) were classified as “chaperone-related proteins”, which was designated in the present study, including proteins with chaperone or catalytic activity for protein folding.

Next, the cDNA clones encoding HSP family members were completely sequenced, resulting in identification of nine genes (Table 2). These clones were designated as in Table 2, based on their deduced protein homology with HSP family members of other vertebrates. Among them, two types having different sequences were found each in *Hsp90* and *Hsc70*. They were designated as *Hsp90βa* and *Hsp90βb*, and *Hsc70a* and *Hsc70b*, respectively. Both *Hsp90βa* and *Hsp90βb* cDNAs contained complete open reading frame (ORF), and their deduced amino acid sequences shared 97.7% identity with each other. The two proteins contained the conserved C-terminal tetrapeptide motif, EEVD (data not shown), which plays an important role in cofactor binding mediated by a tetratricopeptide

repeat (TPR) domain (Scheufler *et al.*, 2000). A BLAST search indicated that both proteins had the highest amino acid identity with Atlantic salmon *Salmo salar* Hsp90β (Table 2). Meanwhile, Hsc70b cDNA contained an incomplete ORF, and its deduced protein lacked 121 amino acid residues from the N-terminus compared with that of Hsc70a cDNA. The cDNA sequence of Hsc70a was identical to that of the genomic clone reported previously as rainbow trout *Hsc71* (Zafarullah *et al.*, 1992). For *Hsp70*, one clone was isolated and identified as *Hsp70a*, which was cloned from a juvenile rainbow trout in the preceding chapter. The two *Hsp70a* proteins derived from different sources showed only one amino acid variation, namely, T429I (data not shown). For *BiP/Grp78*, one incomplete cDNA clone, which lacked the 5' end, was isolated. The deduced protein possessed a C-terminal tetrapeptide KDEL (data not shown), which is conserved in *BiP/Grp78* and required for retrieval of *BiP/Grp78* molecules that leave the endoplasmic reticulum (ER)

(Munro and Pelham, 1987). Besides those described above, cDNAs encoding chaperonin containing TCP1 subunit 8 (Cct8), Hsp47 and DnaJ homolog were identified. Cct8 and Hsp47 cDNAs contained complete ORFs, whereas DnaJ homolog cDNA was a partial fragment containing putative intron(s). The deduced Hsp47 protein contained an RDEL tetrapeptide motif at the C terminus (data not shown) that acts as an ER-retention signal (Sato *et al.*, 1996).

Section 2. Accumulation of HSP mRNAs in unstressed and heat-shocked RTG-2 cells

As a result of the preceding section, DNA sequence information for several *HSPs* became available. Based on this information, it is possible to design probes and primers specific to respective genes. By using these probes and primers, this section describes the mRNA expression profiles of *HSPs* and the quantitative analysis of their mRNA

Table 3. Primers used for synthesis of DIG-labeled probes

Gene	Accession no.	Primer sequence ^a	Nucleotide positions	Product length (bp)
<i>Hsp90βa</i>	AB196457	F: 5'-AATGGGTAACCTGGTCAGTG-3' R: 5'-CTGAATACAGACAGGTCTGA-3'	2301-2600	300
<i>Hsp90βb</i>	AB196458	F: 5'-GTCTCAAACCTACACACCTG-3' R: 5'-CTACATAGCTACCGGTCCAA-3'	2231-2510	280
<i>Grp78</i>	AB196459	F: 5'-TCTGGAGTGGCACAGATGTA-3' R: 5'-CATGTTACCCTTCACCCAGA-3'	1911-2170	260
<i>Hsp70a</i>	AB176854	F: 5'-ACCACGAAATTGGGGAGAAA-3' R: 5'-TGCAATGTCCAACAATGAAA-3'	2544-2756	213
<i>Hsp70b</i>	AB176855	F: 5'-AGAGATTGACTAAAGTGAGGGAT-3' R: 5'-ACATTTTATTTGCAATGTCC-3'	2012-2174	163
<i>Hsc70a</i>	AB196460	F: 5'-ACCTCCCCCTAACAAGCAAA-3' R: 5'-AGGCATTGTGACAAAGGCAG-3'	2091-2310	220
<i>Hsc70b</i>	AB196461	F: 5'-CCACCATTGAGGAAGTCGAT-3' R: 5'-CAGGACTCAAAATGTAGACA-3'	1601-1810	210
<i>Cct8</i>	AB196462	F: 5'-AAACACTGGGGCATCAAAC-3' R: 5'-ATGTCATCGCTCCTTCCATC-3'	1559-1856	298
<i>Hsp47</i>	AB196463	F: 5'-CCAGGAAATGGCACATGTAT-3' R: 5'-TATAAGCATGCTGTCTGGGTC-3'	1381-1670	290
<i>β-actin</i>	AB196465	F: 5'-TGTACCCATCCCAAACGACC-3' R: 5'-TCCTCAGCTGCATGATAGAA-3'	1201-1490	290

^a F, forward primer; R, reverse primer.

accumulation levels before and after heat stress.

Materials and Methods

RNA preparation

Control RTG-2 cells were cultured at 20°C as described in Section 2 of Chapter I. The culture dishes were sealed with Parafilm and immersed into a water bath at 28°C for 3 h. This heat-shock condition gave rise to the highest accumulation levels of Hsp70 mRNAs in Chapter I. Total RNA was isolated from the control and heat-shocked RTG-2 cells with TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. For real-time RT-PCR, 20 μg of each RNA was incubated at 37°C for 20 min with 10 U RNase-free DNase I (Takara Bio) in a 50-μl reaction volume containing 20 U RNase inhibitor (Toyobo). RNAs were extracted with phenol:chloroform:isoamyl alcohol (25:24:1 mixed, pH5.2; Nacalai Tesque, Kyoto, Japan) followed by ethanol precipitation.

Northern blot analysis

Five micrograms of total RNA were separated on a 1% agarose-formaldehyde gel and capillary transferred to Hybond N+ nylon membranes (Amersham Biosciences) with 10 × SSC. RNAs were UV-crosslinked to the membranes, which were subsequently hybridized at 68°C for 1 h in a PerfectHyb hybridization solution (Toyobo) containing DIG-labeled DNA probes. The probes were labeled with a PCR DIG Probes Synthesis kit (Roche Diagnostics) using primers specific to 3'-UTR of each gene (Table 3). The hybridized membranes were washed twice with 2 × SSC plus 0.1% SDS at 68°C for 5 min, and then twice with 2 × SSC plus 0.1% SDS at 68°C for 15 min. The chemiluminescent detection of the probes was performed with a DIG Luminescent Detection kit (Roche Diagnostics) according to the manufacturer's instruction. Positive signals were detected by using a luminescent image analyzer (LAS-1000 mini; Fuji Photo Film, Tokyo, Japan).

Table 4. Primers used for real-time RT-PCR

Gene ^a	Primer sequence ^b	Nucleotide positions	Product length (bp)
<i>Hsp90βa</i>	F: 5'-AATGGGTAACCTGGTCAGTG-3' R: 5'-CTGAATACAGACAGGTCTGA-3'	2301–2600	300
<i>Hsp90βb</i>	F: 5'-AATGCTGTCTCAAACCTACACACC-3' R: 5'-ATACCATAAAAGGACACCGACCA-3'	2225–2335	111
<i>Grp78</i>	F: 5'-TCTGGAGTGGCACAGATGTA-3' R: 5'-CATGTTACCCTTCACCCAGA-3'	1911–2170	260
<i>Hsp70a</i>	F: 5'-CGGGAGTTGTAGCGATGAGA-3' R: 5'-CTTCCTAAATAGCACTGAGCCATAA-3'	2495–2634	140
<i>Hsp70b</i>	F: 5'-AGGCCCAACCATTGAAGAGA-3' R: 5'-GCAATGTCCAGCAATGCAATA-3'	1997–2163	167
<i>Hsc70a</i>	F: 5'-ACCTCCCCCTAACAAGCAAA-3' R: 5'-AGGCATTGTGACAAAGGCAG-3'	2091–2310	220
<i>Hsc70b</i>	F: 5'-GCCCAATCTGTAGTAAAGCCAAG-3' R: 5'-CTCCAGGACTCAAATGTAGACAAA-3'	1669–1813	145
<i>Cct8</i>	F: 5'-CTCTACCCTTTTGACCACCTAACC-3' R: 5'-CTCCTTCCATCACACAGTAACCAC-3'	1698–1847	150
<i>Hsp47</i>	F: 5'-CCAGGAAATGGCACATGTAT-3' R: 5'-TATAAGCATGCTGTCTGGGGTC-3'	1381–1670	290
<i>β-actin</i>	F: 5'-TGGGGCAGTATGGCTTGTATG-3' R: 5'-CTCTGGCACCCCTAATCACCTCT-3'	1624–1788	165

^a Accession numbers for genes are in Table 3.

^b F, forward primer; R, reverse primer.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and ABI PRISM 7000 Sequence Detection System (Applied Biosystems). One-step RT-PCR was performed in a 25-μl total reaction volume including 12.5 μl of 2 × QuantiTect SYBR Green RT-PCR master mix, 0.25 μl QuantiTect RT mix, 50 ng RNA template, and 0.2 μM each of target specific primers designed to amplify a part of 3'-UTR of each gene (Table 4). To quantify each target transcript, a standard curve was constructed with serial dilutions of total RNA extracted from heat-shocked (28°C, 3 h) RTG-2 cells for every set of primers. Reverse transcription was performed at 50°C for 30 min, and thermal cycling conditions were as follows: 95°C for 15 min; and 40 cycles of 95°C for 5 s and 60°C for 31 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of

the PCR product, as displayed by a single peak (data not shown). The control, containing all the reaction components except for the template, was included in all experiments. The amount of each HSP mRNA was then normalized to the abundance of a housekeeping gene, *β-actin*. To evaluate the validity of using *β-actin* as an internal standard, possible changes in the amounts of *β-actin* mRNA were tested before and after heat shock by using λ poly(A)⁺ RNA-A (Takara Bio) as an external standard. The normalized values of each HSP mRNA in heat-shocked cells were divided by those in controls. Student's unpaired *t* test was used for group comparisons.

Results

To investigate whether the cloned HSP genes are actually transcribed in rainbow trout cells,

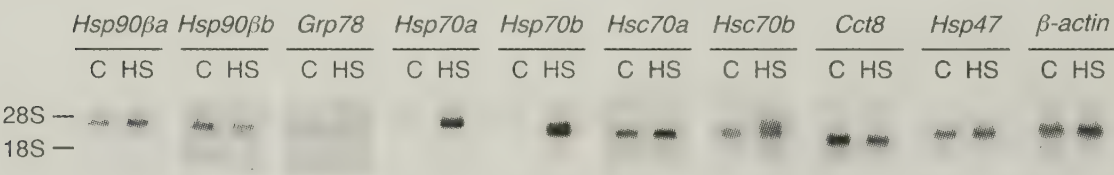


Fig. 7. mRNA accumulation levels of HSPs in control (C) and heat-shocked (HS) RTG-2 cells as revealed by Northern blot analyses. The probes are complementary to sequences in 3'-UTR of respective genes. The positions of 28S and 18S rRNAs are indicated on the left as size markers. The blots are representatives of three independent experiments.

Table 5. mRNA accumulation levels of HSPs relative to those in control

Gene	Control (20 °C) ^a	Heat shock (28 °C, 3 h) ^a	Significance level ^b	P value ^b
<i>Hsp90βa</i>	1.0 (0.5)	1.0 (0.6)	0.05	0.99 ^c
<i>Hsp90βb</i>	1.0 (0.4)	1.1 (0.4)	0.05	0.77 ^c
<i>Grp78</i>	1.0 (0.4)	1.1 (0.5)	0.05	0.78 ^c
<i>Hsp70a</i>	1.0 (0.3)	480 (170)	0.001	<0.001 ^d
<i>Hsp70b</i>	1.0 (0.4)	510 (150)	0.001	<0.001 ^d
<i>Hsc70a</i>	1.0 (0.3)	1.3 (0.4)	0.05	0.0083 ^d
<i>Hsc70b</i>	1.0 (0.3)	2.8 (0.8)	0.001	<0.001 ^d
<i>Cct8</i>	1.0 (0.6)	0.7 (0.2)	0.05	0.12 ^c
<i>Hsp47</i>	1.0 (0.3)	1.6 (0.1)	0.001	<0.001 ^d

^a Values are means (SD); n = 6.
^b The Student's unpaired *t* test was used for group comparisons.
^c Two-sided test.
^d One-sided test.

Northern blot analysis was performed using DNA probes specific to 3'-UTR of respective genes. In this regard, the DnaJ homolog gene was excluded from this analysis because its cDNA was incomplete where 3'-UTR was uncertain. Meanwhile, *Hsp70b* isolated in Chapter I was included. Consequently, single bands were detected for all genes examined except *Hsp70s* where two mRNA species having different sizes were detected in heat-shocked cells irrespective of *Hsp70a* and *Hsp70b* as observed in Chapter I (Fig. 7). Therefore, it was concluded that the cloned genes are actually transcribed in RTG-2 cells.

Although *Hsp70a* and *Hsp70b* transcripts were apparently induced by heat shock, changes in mRNA accumulation levels were ambiguous for the other HSP family members (Fig. 7). Thus, to accurately determine the changes, quantitative RT-PCR analysis was performed. In this regard, the β -actin gene was used as an internal standard for normalizing the mRNA accumulation levels of HSP genes, because no significant differences were found in the β -actin mRNA levels between control and heat-shocked cells ($P=0.36$, Student's *t* test, two-sided), at least under the heat-shock conditions applied here (data not shown). Consequently, significantly increased after heat shock were the mRNA accumulation levels of five HSP genes, namely, *Hsp70a*, *Hsp70b*, *Hsc70a*, *Hsc70b*, and *Hsp47* (Table 5). In particular, the accumulation levels of *Hsp70a* and *Hsp70b* mRNAs were dramatically increased after heat shock, and the mean values in heat-shocked cells were 480- and 510-fold of those in controls, respectively (Table 5). The increased levels of *Hsc70a*, *Hsc70b* and *Hsp47* mRNAs were 1.3- to 2.8-fold on average after heat shock and considerably lower than those of *Hsp70* mRNAs (Table 5). Further, when *Hsc70a* and *Hsc70b* were compared, the increased mRNA level of *Hsc70b* was more remarkable than that of *Hsc70a*, as suggested by a statistical analysis (Table 5). On the other hand, there were no differences in mRNA levels of the other four HSP genes between control and heat-shocked cells with a significance level of 0.05 (Table 5).

Section 3. Discussion

In this chapter, multiple genes encoding HSP family members were isolated, and their mRNA accumulation levels were comprehensively compared in RTG-2 cells before and after heat shock. To accurately compare the mRNA levels of these HSP genes, real-time RT-PCR was used.

In preliminary experiments, it was found that the mRNA accumulation level of the β -actin gene was reduced in RTG-2 cells after 24-h exposure to heat shock at 25°C (data not shown). Accordingly, it was considered that the ratios of mRNAs of the HSP genes to those of the house-keeping gene would be relatively high in the heat-shocked cells, and utilization of the cells as a source of a cDNA library was attempted to isolate HSP genes as many as possible. As expected, multiple HSP genes were identified by arbitrarily isolating cDNAs from the RTG-2 cells (Table 1). Among the cloned genes, two cDNAs having different sequences were found in each of *Hsp90* and *Hsc70*. These were suggested to be paralogous genes as in the case of rainbow trout *Hsp70s* in Chapter I. Although only one clone could be identified each for the other HSP family genes in the present study, each may have a possible paralog because it has been inferred that most of the rainbow trout genes are duplicated (Ohno *et al.*, 1968; Palti *et al.*, 2004).

Sequence analyses of the two *Hsp90* cDNAs showed that both deduced proteins were highly homologous to Atlantic salmon *Hsp90 β* . In vertebrates, two major *Hsp90* isoforms are present in the cytosol, namely, *Hsp90 α* and *Hsp90 β* (Sreedhar *et al.*, 2004). *Hsp90 α* is highly heat-inducible, whereas *Hsp90 β* is expressed constitutively at a high level at normal temperatures and its expression is weakly dependent on heat shock (Scheibel and Buchner, 1997). The two *Hsp90* genes in the present study, designated *Hsp90 β a* and *Hsp90 β b*, were constitutively expressed in RTG-2 cells irrespective of heat-shock treatment (Fig. 7 and Table 5), suggesting that both genes actually encode a β isoform of *Hsp90*. On the other hand, Sathiyaa *et al.* (2001) isolated a partial fragment of rainbow trout *Hsp90* cDNA, showing that its transcripts were

induced by heat shock in hepatocytes as revealed by Northern blot analysis. The reported partial sequence is not identical to the Hsp90s in the present study, raising the possibility that there exists at least one α isoform of Hsp90 besides the two β isoforms in rainbow trout. This possibility is also supported by the result that an α isoform of Hsp90 has been isolated from another salmonid, the Chinook salmon *Oncorhynchus tshawytscha* (Palmisano *et al.*, 1999).

In contrast to the other HSP family members examined, the accumulation levels of Hsp70 mRNAs were dramatically increased after heat shock (Fig. 7 and Table 5). In Northern blot analysis (Fig. 7), two mRNA species having different sizes were detected in heat-shocked cells irrespective of *Hsp70a* and *Hsp70b* as described in Chapter I. Since nearly the same regions were detected for the two genes in Northern blot and real-time RT-PCR analyses, it is inferred that the accumulation levels of Hsp70 mRNAs in the latter analysis were those of integrated values of the two mRNA species detected in the former analysis. In any case, given the magnitude of the changes in the mRNA levels, it is strongly suggested that *Hsp70s* are the most useful biomarkers of heat stress among HSP family members in fish, at least in rainbow trout.

The study in this chapter demonstrated that there exist two *Hsc70s* in rainbow trout cells like *Hsp70s* in Chapter I. Zafarullah *et al.* (1992) isolated a genomic clone encoding Hsc70 from rainbow trout and designated it as *Hsc71*, which corresponds to the *Hsc70a* in this chapter (Table 2). Hsc70 is a member of the HSP70 family and exhibits constitutive expression (Hightower and Leung, 1997). It has been reported that Hsc71 mRNA levels did not significantly increase after heat shock in RTG-2 cells (Zafarullah *et al.*, 1992), and similar results were obtained by Northern blot analysis in Chapter I. However, by using quantitative RT-PCR analysis, it was demonstrated that the accumulation levels of both *Hsc70a* and *Hsc70b* mRNAs were significantly increased after heat shock in the present study (Table 5). Considering that several putative HSEs have been found in the promoter region of *Hsc71* (Zafarullah *et al.*, 1992), it is likely that there exist functional HSEs for the rainbow

trout *Hsc70s*. Furthermore, since the mRNA accumulation levels in heat-shocked cells differed between *Hsc70a* and *Hsc70b*, the two genes may have promoters with different activities.

Cct8, one of the subunits of the CCT complex abundant in eukaryotic cytosol, is a member of the chaperonin/HSP60 family (Kubota and Willison, 1997). Until now, the heat shock inducibility of CCT subunits has not been investigated in fish. In rainbow trout RTG-2 cells, quantitative RT-PCR analysis showed that there was no significant increase in the Cct8 mRNA levels after heat shock (Table 5). Likewise, it has been reported that no significant increase of CCT subunits was detected in response to heat shock at protein levels in both HeLa and mouse BALB/3T3 cells (Kubota *et al.*, 1999). However, as discussed by Kubota *et al.* (1999), CCT may be induced depending on the type of stress, cells, organisms and other environmental conditions.

In RTG-2 cells, the mRNA accumulation levels of *Hsp47* were significantly increased after heat shock (Table 5). It is known that *Hsp47* expression is induced by heat shock at the transcriptional level in zebrafish embryos (Pearson *et al.*, 1996) and chicken embryonic fibroblasts (Hirayoshi *et al.*, 1991). In vertebrate cells, Hsp47 is located in the lumen of ER (Nagata, 1997), as is BiP/Grp78 (Gething, 1997b). In rainbow trout, however, the cellular localizations of Hsp47 and Grp78 have not been determined. Considering that rainbow trout Hsp47 and Grp78 had ER retention signals (data not shown), it is suggested that both proteins are located in the ER lumen like their counterparts from other vertebrates. In addition, consistent with mammalian cells (Gething, 1997b), the results in this chapter indicate that rainbow trout Grp78 mRNA was constitutively expressed in unstressed cells (Fig. 7 and Table 5).

The enhanced synthesis has been observed with polypeptides having 100, 87, 70, 68, 60, 39, and 27 kDa in RTG-2 cells exposed to heat shock at 28°C (Mosser *et al.*, 1986). Under the same heat-shock conditions, the enhanced syntheses of a similar set of HSPs have also been found by another research group (Kothary and Candido, 1982), although there were slight differences in the polypeptide size together

with an additional polypeptide having 32 kDa. Judging from the molecular sizes, it is thought that the above-mentioned polypeptides belong to either of the HSP100, HSP90, HSP70, HSP60, HSP40, and HSP27 families. Among these, the genes encoding the HSP90, HSP70, HSP60, and HSP40 families were cloned in this chapter. Unfortunately, however, cDNAs encoding HSP100 and HSP27 families could not be found in 200 cDNAs arbitrarily isolated in the present study.

Chapter III. Cloning and characterization of HSF1

The expression of HSP genes is regulated by heat-shock transcription factors (HSFs) that bind to a specific *cis*-acting element, namely, HSE (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995). In vertebrates, genes encoding four types of HSFs, HSF1-4, have been cloned. Among the HSF family members, HSF1 is the principal transcriptional factor activated by exposure to stresses such as heat shock, and this protein is known to form homotrimers that bind DNA (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995).

During evolution, fish have adapted to live in various ambient temperatures. Reflecting such adaptations, the threshold temperature for HSP induction differs between cold- and warm-adapted fish. For example, HSPs are induced in the 26–30 °C range in rainbow trout RTG-2 cells (Mosser *et al.*, 1986), whereas HSP70 is induced in the 35–37 °C range in zebrafish tissues (Råbergh *et al.*, 2000). However, little is known about the molecular mechanisms underlying the difference in HSP induction temperatures among fish species. To date, Råbergh *et al.* (2000) reported the isolation of one *HSF1* cDNA from zebrafish, which is a warm-adapted fish. Although a cDNA fragment encoding an HSF has also been cloned from bluegill sunfish *Lepomis machrochirus* (Råbergh *et al.*, 2000), a full-length HSF1 cDNA clone has not been isolated from any fish other than zebrafish. Some authors (Airaksinen *et al.*, 1998; Le Goff and Michel, 1999) have reported the presence of a protein that possesses HSF1-like activity in rainbow trout; however, a gene encoding HSF1 itself has not been identified in this cold-adapted fish.

Thus, the purpose of the study was to isolate a cDNA clone encoding rainbow trout HSF and to characterize its translation products. This chapter describes the existence of two distinct HSF1 isoforms in rainbow trout and the evidence for heterotrimer formation of these isoforms *in vitro*.

Section 1. cDNA cloning and genomic organization of HSF1

As a result of the preceding chapters, it was shown that some rainbow trout *HSPs*, especially *Hsp70s*, are induced at the transcriptional level by heat stress. This suggests that cells of rainbow trout have an HSF as do other vertebrates. Thus, to demonstrate the existence of the transcription factor, this section describes the cDNA cloning of rainbow trout HSF and its gene structure.

Materials and Methods

Cell culture and animals

RTG-2 cells were cultured at 15°C as described in Section 2 of Chapter I. Rainbow trout (3 months old), which were used to extract total RNA for RT-PCR, were obtained from the Nikko Station of the National Research Institute of Fisheries Science (Tochigi Prefecture, Japan) and reared on a commercial diet at 15°C.

cDNA cloning

A random primed λ ZAPII cDNA library was constructed by using a λ ZapII predigested *EcoRI*/CIAP-treated vector kit (Stratagene, La Jolla, CA, USA) with RNA isolated from RTG-2 cells as described below. Approximately 1.2×10^6 plaques were screened at 2×10^5 plaques per 140×100 -mm plate by hybridization of duplicate nitrocellulose membranes with a 2.7-kb fragment of chicken HSF1 cDNA (Nakai and Morimoto, 1993) as a probe. The membranes were soaked in $2 \times \text{SSC}$ for 5 min, prehybridized at 42°C for 2 h with hybridization buffer [$6 \times \text{SSC}$, $1 \times \text{Denhardt's}$ solution, 0.15% SDS, and $100 \mu\text{g/ml}$ denatured calf thymus DNA (Invitrogen)], and hybridized with a ^{32}P -labeled DNA probe in the same buffer at 42°C for 16 h. The membranes were then rinsed twice with $2 \times \text{SSC}$.

plus 0.1% SDS at room temperature for 5 min per rinse, washed twice in 2 × SSC plus 0.1% SDS at 50 °C for 20 min per wash, dried, and exposed to X-ray film for 2 days. Positive clones were isolated through three rounds of screening. Phagemid pBluescript SK (–) was excised from purified plaques with helper phage according to the manufacturer’s instructions.

The 5’- and 3’- termini of rainbow trout HSF cDNAs were isolated by rapid amplification of cDNA ends (RACE). A directional cDNA library constructed in Section 1 of Chapter II was used as the PCR template. For 5’-RACE, the first PCR was performed with the M13 reverse primer (5’-AGCGG ATAACAATTTTCACACAGG-3’) as a sense primer and a rainbow trout *HSF1*-specific primer (5’-ATCT TTCTCTTCATCCCCAGGACT-3’) as an antisense primer. The nested PCR was performed with the T7 promoter primer (5’-TAATACGACTCACTATAG GG-3’) as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5’-TGCCTTTTATGTTCTGCACGA-3’ ; for *HSF1b*, 5’-CCTCCCTCCACAGAGCTTCA-3’) as antisense primers. For 3’-RACE, the first PCR was performed with the M13 forward primer (5’-CCCAGTCACGACGTTGTAAAACG-3’) as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5’-GAAGCAGCTTGTCCAGTACACTAA-3’; for *HSF1b*, 5’-GAAGCAGCTGGTCCAGTACACCT

C-3’) as antisense primers. The nested PCR was performed with the SP6 promoter primer (5’-ATTTAGGTGACACTATA-3’) as a sense primer and *HSF1*-specific primers (for *HSF1a*, 5’-CGGAC CTCCCCACTCTGCTGGAGA-3’; for *HSF1b*, 5’-TC CCCACTCTGCTGGAGCTGGAGG-3’) as antisense primers. The amplified products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA).

Sequence determination

Nucleotide sequences were determined from both strands by a 373A DNA sequencer (Applied Biosystems) and a Thermo Sequenase II Dye Terminator Cycle Sequencing kit (Amersham Biosciences).

Phylogenetic analysis

A phylogenetic tree was constructed as described in Section 1 of Chapter I. The setting parameters were as follows: MATRIX, BLOSUM; GAOPEN, 10.0; GAPEXT, 0.05; GAPDIST, 8; MAXDIV, 40; ENDGAPS, off; NOPGAPS, off; NOHGAPS, off.

Southern blot analysis

Genomic DNA was isolated from RTG-2 cells by a GenomicPrep Cells and Tissue DNA Isolation kit (Amersham Biosciences) according to the

Table 6. Comparison of rainbow trout HSF1s with other vertebrate HSF1

Species ^a	Percentage identity (%) ^b					
	ORF	I ^c	II ^c	III ^c	IV ^c	V ^c
rtHSF1a	100	100	100	100	100	100
rtHSF1b	86.4	89.8	96.0	83.9	97.0	78.8
zHSF1	68.4	89.8	73.3	61.6	78.8	50.5
cHSF1	55.7	88.0	78.7	40.3	39.4	40.0
hHSF1	55.3	85.2	78.7	41.8	42.4	39.1
rtHSF1b	100	100	100	100	100	100
zHSF1	69.5	95.4	74.7	59.9	81.8	52.1
cHSF1	55.9	92.6	81.3	38.0	42.4	40.2
hHSF1	56.4	90.7	81.3	40.0	48.5	41.7

^a rt, rainbow trout; z, zebrafish; c, chicken; h, human.
^b The percentage amino acid identity was calculated by the ALIGN program in LASERGENE software.
^c The five regions, I–V, are indicated in Fig. 10.

manufacturer's instructions. Ten micrograms of genomic DNA was digested with *Bam*HI, *Eco*RI, or *Hind*III, resolved by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (Hybond N+; Amersham Biosciences). The membranes were hybridized for 1 h in PerfectHyb hybridization solution (Toyobo) with DIG-labeled DNA probes. As the probes, the 3'-UTRs of *HSF1a* and *HSF1b* were labeled by a PCR DIG Probes Synthesis kit (Roche Diagnostics). The probed regions of *HSF1a* and *HSF1b* correspond to nucleotides 1651–2027 and 1691–2052, respectively. The hybridized membranes were washed twice with $2 \times$ SSC plus 0.1% SDS at room temperature for 5 min, and then twice with $0.1 \times$ SSC plus 0.1% SDS at 68°C for 15 min. The chemiluminescent detection of the probes was performed with a DIG luminescent detection kit (Roche Diagnostics) according to the manufacturer's instructions. The positive signals were detected by exposure on Hyperfilm-MP (Amersham Biosciences).

Results

By screening an RTG-2 cDNA library using a chicken HSF1 cDNA probe, two positive clones, named C1 and C2, were isolated. Sequence analysis revealed that these two clones encoded distinct isoforms of HSF. Clone C1 was a partial cDNA containing an insert of 983 nucleotides encoding the DNA-binding domain of HSF, whereas clone C2 contained an insert of 2771 nucleotides including introns and an ORF encoding 513 amino acids.

By using 5'- and 3'-RACE, the full-length cDNAs of clones C1 and C2 without introns were determined to be 2083 bp and 2142 bp, respectively. Clones C1 and C2 were predicted to encode proteins of 501 and 513 amino acids, respectively (Fig. 8). Phylogenetic analysis indicated that the two proteins belong to the HSF1 cluster (Fig. 9). Accordingly, clones C1 and C2 are referred to hereafter as rainbow trout HSF1a and HSF1b, respectively.

The sequence identity between the two predicted proteins was 86.4% (Table 6). By contrast, the whole ORF of the rainbow trout HSF1s showed low homology to those of other vertebrate HSF1s. For example, rainbow trout HSF1a and HSF1b

showed 55.3% and 56.4% identity to human HSF1, respectively (Table 6).

Next, the structural features of HSF1a and HSF1b were examined in comparison to those of other vertebrate HSF1s. HSF1 has been reported to contain conserved regions referred to as the DNA-binding domain (DBD), and the amino-terminal and carboxyl-terminal hydrophobic heptad repeats (HR-A/B and HR-C, respectively) (Morimoto, 1998; Pirkkala *et al.*, 2001; Wu, 1995). Multiple sequence alignment demonstrated that both of the rainbow trout HSF1s contained these conserved domain structures (Fig. 8), as shown schematically in Fig. 10.

Region I (DBD) of the rainbow trout HSF1s showed high similarity to the corresponding region of zebrafish, chicken, and human HSF1 (Table 6); for instance, DBD of rainbow trout HSF1b shared 90.7% identity with that of human HSF1. By contrast, regions II (HR-A/B) and IV (HR-C) of the rainbow trout HSF1s showed less similarity to the corresponding regions of other vertebrate HSF1s (Table 6). However, the actual heptad repeats of hydrophobic amino acids are conserved across the whole HSF1 family (Fig. 8). In addition, two KRK tripeptides, which are conserved among characterized HSF1 family members, were identified in both of the rainbow trout HSF1s (Fig. 8). In contrast to the highly conserved regions described above, regions III and V of rainbow trout HSF1s showed low similarity to the counterparts of other vertebrate HSF1s (Table 6). Notably, region V showed low similarity across the HSF1 family, even between rainbow trout HSF1a and HSF1b (78.8% identity; Table 6).

The genomic organization of the rainbow trout *HSF1s* were examined by Southern blot analysis using the 3'-UTRs of *HSF1a* and *HSF1b* as probes. These two probes showed different hybridized patterns (Fig. 11), demonstrating that each HSF1 is encoded by a distinct gene in the genome.

Section 2. mRNA expression pattern of HSF1 in various tissues

In the preceding section, it was shown that two distinct genes encoding HSF1 are present in

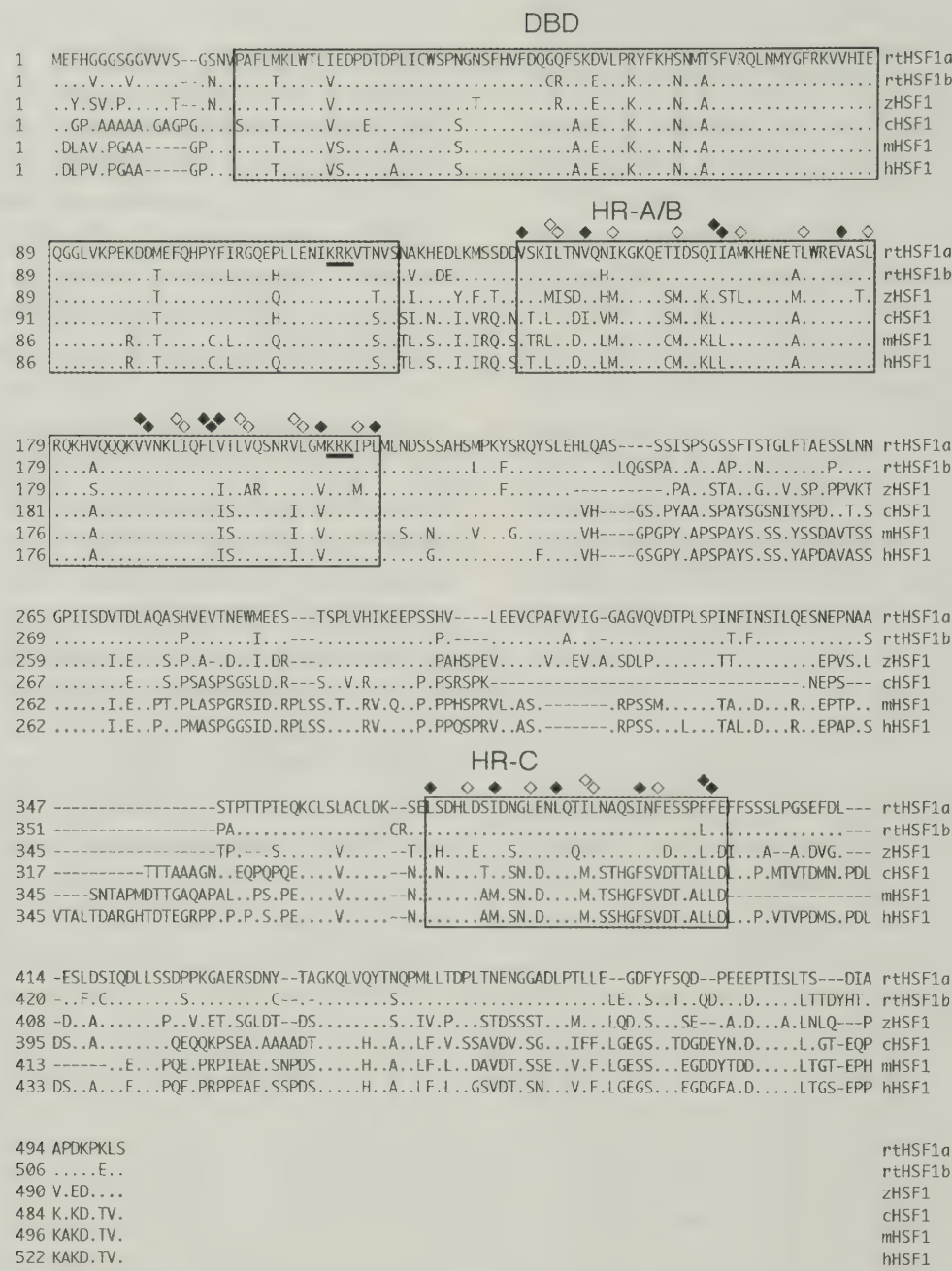


Fig. 8. Comparison of the predicted amino acid sequences of rainbow trout (rt) HSF1a and HSF1b with the sequences of zebrafish (z), chicken (c), mouse (m) and human (h) HSF1. The three domain structures, DBD, HR-A/B and HR-C, are boxed. Open and filled diamonds indicate the repeats of hydrophobic amino acids. The underlined KRK tripeptides are putative nuclear localization signals (NLSs). The numbers on the left indicate the amino acid positions of each protein.

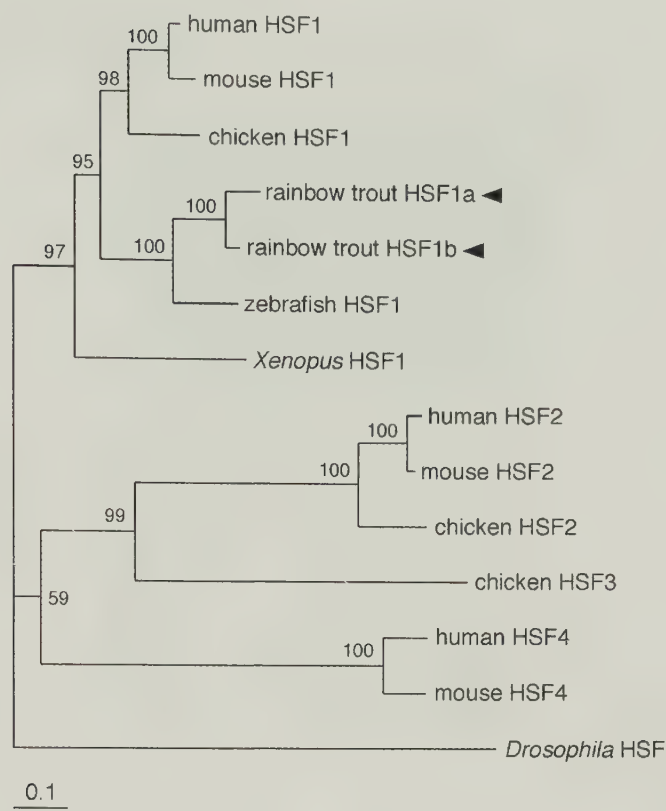


Fig. 9. Phylogenetic tree of the vertebrate HSF family based on the amino acid sequences. The tree was calculated by neighbor joining, with *Drosophila* HSF used as an outgroup. Arrowheads indicate the position of rainbow trout HSF1a and HSF1b. Numbers at the nodes indicate the percentage of bootstrap values (1000 replicates). The scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site. GenBank accession numbers for the sequences are as follows: human HSF1 (M64673), HSF2 (M65217), HSF4 (D87673); mouse HSF1 (X61753), HSF2 (X61754), HSF4 (AB029350); chicken HSF1 (L06098), HSF2 (L06125), HSF3 (L06126); *Xenopus* HSF1 (L36924); zebrafish HSF1 (AB062117); rainbow trout HSF1a (AB062548), HSF1b (AB062549); *Drosophila* HSF (M60070).



Fig. 10. Schematic representation of HSF1 domain structures. The three regions of identity are denoted region I, corresponding to DBD; region II, corresponding to the amino-terminal hydrophobic heptad repeat (HR-A/B); and region IV, corresponding to the carboxyl-terminal hydrophobic heptad repeat (HR-C). Regions III and V roughly correspond to domains of mammalian HSF1, namely, the regulatory and transactivation domains, respectively.

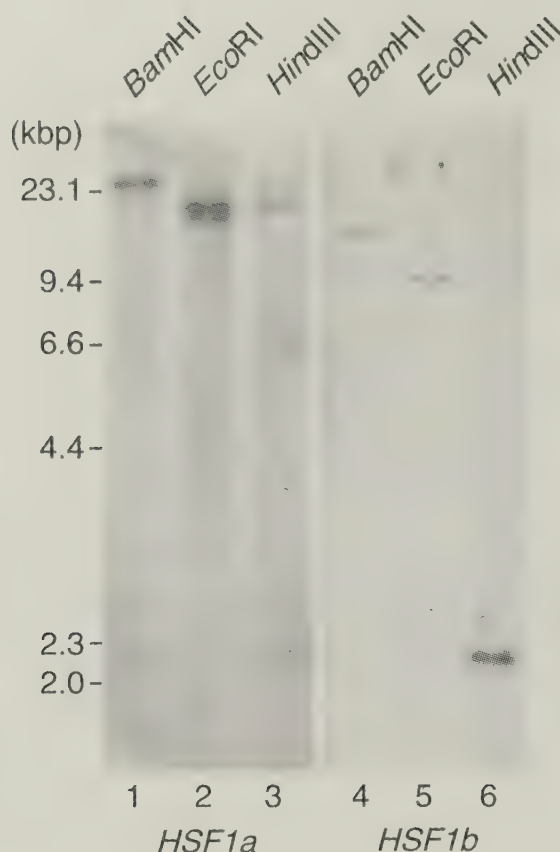


Fig. 11. Genomic Southern blot analysis of rainbow trout *HSF1*s. In the left panel, hybridization was carried out with a DIG-labeled *HSF1a* probe (a 377-base fragment of the 3'-UTR of *HSF1a* cDNA), whereas in the right panel, hybridization was carried out with a DIG-labeled *HSF1b* probe (a 362-base fragment of the 3'-UTR of *HSF1b* cDNA). λ DNAs digested with *HindIII* were used as molecular markers and are indicated on the left.

rainbow trout cells. This raises the question that the two genes may have distinct expression patterns in various tissues. To answer this question, this section describes the expression of *HSF1* mRNAs in various tissues of rainbow trout.

Materials and Methods

Isolation of RNA and RT-PCR analysis

Total RNA was isolated from RTG-2 cells and rainbow trout (6 months old) tissues with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 5 μ g of total RNA by using a SuperScript First-Strand Synthesis System for

RT-PCR kit (Invitrogen). After reverse transcription, DNase I digestion was performed to eliminate residual genomic DNA from the RNA samples. PCR was carried out in a total volume of 50 μ l with 0.5 μ l of cDNA synthesis mixture containing HotStarTaq DNA Polymerase (Qiagen) in an automated thermal cycler (model 2400; PerkinElmer, Wellesley, MA, USA). PCR consisted of one initiation cycle of 15 min at 95°C, amplification cycles of 0.5 min at 94°C, 0.5 min at 50°C and 0.5 min at 72°C, and one termination cycle of 1 min at 72°C, with 35 cycles in total for *HSF1a* and *HSF1b* and 30 for *Hsc70*. *Hsc70* cDNA was amplified as a positive control, because *Hsc70* mRNA is constitutively expressed in different rainbow trout tissues (Zafarullah *et al.*, 1992) and

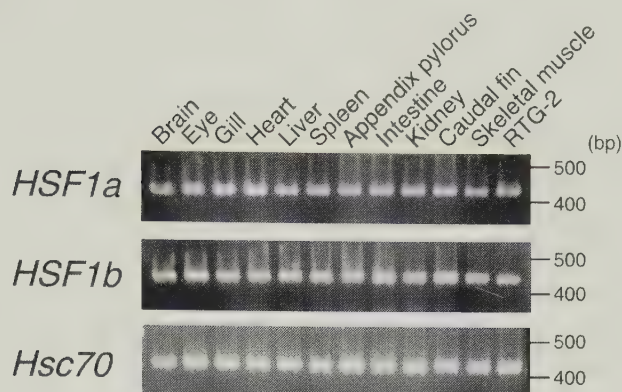


Fig. 12. RT-PCR analysis of the *HSF1a* and *HSF1b* genes in rainbow trout RTG-2 cells and tissues. Rainbow trout *Hsc70* was subjected to RT-PCR analysis as a positive control. Molecular size markers are indicated on the right (in base pairs).

in RTG-2 cells as shown in the preceding chapters. The oligonucleotide primers were as follows: *HSF1a* forward, 5'-GAAGCAGCTTGTCAGTACACCAA-3'; *HSF1a* reverse, 5'-TTCCAAGAGCTGAACAAA CCATTG-3'; *HSF1b* forward, 5'-GAAGCAGCTGGT CCAGTACACCTC-3'; *HSF1b* reverse, 5'-GGCTGA ATAAACCATGCCAGTAGC-3'; *Hsc70* forward, 5'-ACATCAGCGACAACAAGAGG-3'; *Hsc70* reverse, 5'-AGCAGGTCCTGGACATTCTC-3'. The amplified products were visualized by ethidium bromide staining.

Results

To determine whether the two *HSF1*s cloned from RTG-2 cells are actually transcribed in rainbow trout, RT-PCR was used to analyze total RNA isolated from unstressed RTG-2 cells and rainbow trout tissues. As a positive control, rainbow trout *Hsc70* cDNA was analyzed. The PCR products were predicted to be 423 bp for *HSF1a*, 439 bp for *HSF1b* and 421 bp for *Hsc70*, and bands corresponding to these sizes were amplified (Fig. 12). *HSF1a* and *HSF1b* transcripts were both detected in all rainbow trout tissues examined, as well as in RTG-2 cells. These bands were not due to contamination by genomic DNA because no bands were amplified in the negative control reactions in which total RNA was used as the template without reverse

transcription (data not shown). Taken together, these results demonstrate that the *HSF1a* and *HSF1b* mRNAs are coexpressed in unstressed rainbow trout cells without tissue specificity.

Section 3. Construction of HSF1 expression vectors

In the preceding section, it was shown that two rainbow trout *HSF1*s are coexpressed in all tissues examined. To further characterize their function, the proteins themselves are required. To this end, this section describes the construction of plasmid vectors that express epitope-tagged HSF1s, and confirmation of the protein expression.

Materials and Methods

Plasmids

To distinguish between *HSF1a* and *HSF1b* in the following experiments, hemagglutinin (HA) tagged *HSF1a* (*HSF1a*-HA) and Protein C tagged *HSF1b* (*HSF1b*-Protein C) were constructed. The coding regions of both *HSF1* cDNAs were amplified with the specific PCR primers possessing a *Hind*III or *Not*I restriction enzyme site. The primers were as follows: *HSF1a* forward, 5'-CCCAAGCTTGATA TGGAGTTCACGGTGG-3'; *HSF1a* reverse, 5'-TATGCGGCCGCGAGGATAATTGGGCTTGTC TGG-3'; *HSF1b* forward, 5'-CCCAAGCTTGATA

ATGGAGTTTTCACGTTGG-3'; *HSF1b* reverse, 5'-TATGCGGCCGCGGATAGTTCGGGCTTGTCTGG-3'. PCR was carried out in a total volume of 50 μ l with KOD-Plus-DNA Polymerase (Toyobo) using 1 μ l of the plasmid RTG-2 cDNA library described in Section 1 of Chapter II as the template. PCR consisted of one initiation cycle of 2 min at 94°C, amplification cycles of 0.25 min at 94°C, 0.5 min at 53.6°C and 1.5 min at 68°C, and one termination cycle of 1 min at 68°C, with 37 cycles in total. The C terminus of HSF1a was fused to an HA epitope tag in plasmid pMH (Roche Diagnostics) at *Hind*III and *Not*I restriction enzyme sites. Likewise, the C terminus of HSF1b was fused to a Protein C epitope tag in plasmid pMX (Roche Diagnostics). In control experiments, pHMlacZ and pXMcZ (Roche Diagnostics), which contain the β -galactosidase gene cloned in-frame with an N-terminal tag of either HA or Protein C, were used.

Coupled *in vitro* transcription and translation

Coupled *in vitro* transcription/translation was performed with a TNT Quick Coupled Transcription

/Translation System (Promega) according to the manufacturer's instructions. For the reaction, 1 μ g each of the plasmids described above was used as a template in a 50- μ l reaction mixture.

Western blot analysis

Ten microliters of *in vitro* translated products were separated by SDS-PAGE on 10% gels and transferred to poly(vinylidene difluoride) (PVDF) membranes (Hybond-P; Amersham Biosciences) by electrophoretic transfer. The membranes were blocked with Tris-buffered saline containing 5% skim milk for 1 h at room temperature. Antibodies against HA or Protein C (Roche Diagnostics) were used to detect epitope-tagged proteins at a working concentration of 1 μ g/ml each. Incubation and washing procedures for these antibodies were performed according to the manufacturer's instructions. An ECL Western blotting analysis system (Amersham Biosciences) was used to detect the epitope-tagged proteins. Positive signals were detected by exposure on Hyperfilm-ECL (Amersham Biosciences).

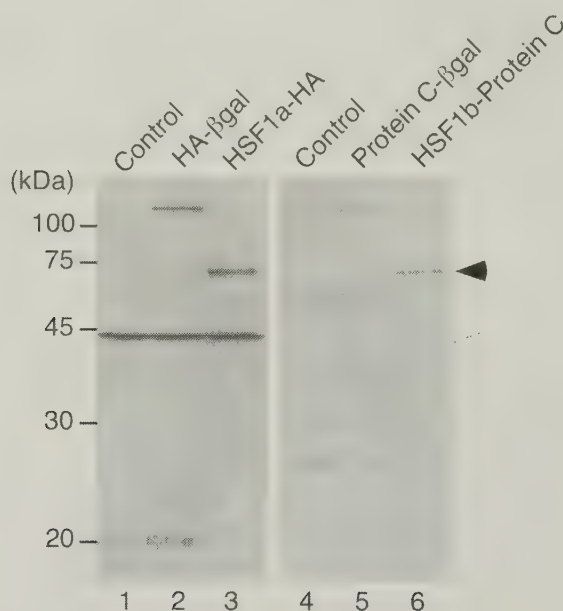


Fig. 13. Western blot analysis of *in vitro* translated expression vectors. Filled arrowhead indicates the position of the epitope-tagged rainbow trout HSF1a and HSF1b (lanes 3 and 6, respectively); open arrowhead indicates nonspecific bands (lanes 1-3). The TNT Quick Master Mix (Promega) used for *in vitro* translations was analyzed as a negative control (lanes 1 and 4), and vectors encoding epitope-tagged β -galactosidase (HA- β gal or Protein C- β gal) were translated *in vitro* as positive controls (lanes 2 and 5).

Results

A coupled *in vitro* transcription/translation assay was performed for the two HSF1s using cDNAs encoding epitope-tagged HSF1 (HSF1a-HA and HSF1b-Protein C) to check for protein expression. As positive controls, cDNAs of epitope-tagged β -galactosidase (HA- and Protein C- β gal) were translated. The reaction mixtures were subjected to Western blotting, and the translated products were detected by antibodies against the epitope tags. Specific translation products were detected in lanes containing those from the HSF1 expression vectors (Fig. 13, lanes 3 and 6). From their migration on the gel, the apparent molecular masses of HSF1a-HA and HSF1b-Protein C were estimated to be 70 kDa and 72 kDa, respectively (Fig. 13, lanes 3 and 6, respectively). These sizes were, however, larger than the expected molecular masses of 57 200 Da for HSF1a-HA and 58 590 Da for HSF1b-Protein C calculated from the predicted amino acid sequences.

Section 4. DNA-binding ability of HSF1

In the preceding section, epitope-tagged HSF1s were successfully synthesized *in vitro*. It is known that specific binding to HSE in the promoter of *HSPs* is a common feature of mammalian and avian HSF1. Do HSF1s of rainbow trout have HSE-binding ability as in the case of other vertebrates? By using the *in vitro* translated HSF1s, this section describes the sequence-specific DNA binding ability of rainbow trout HSF1s.

Materials and Methods

Preparation of whole cell extracts

RTG-2 cells were cultured in a 100-mm dish (Asahi Techno Glass, Funabashi, Japan) at 15°C as described in Section 2 of Chapter I. The dishes were sealed with Parafilm and immersed in a water bath at 25°C for 1 h for heat shock. The cells were harvested, centrifuged, and rapidly frozen at -80°C. The frozen pellets were suspended in extraction buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.1 M KCl, 1 mM dithiothreitol, 20% glycerol]. Protease

inhibitor cocktail (Complete, Mini, EDTA-free; Roche Diagnostics) was added to the extraction buffer at the concentration recommended by the manufacturer. The pellets were homogenized by five freeze-thaw cycles with liquid nitrogen and pipetting. The homogenates were centrifuged at 10,000 *g* at 4°C for 5 min. The supernatants were collected, and the protein concentrations were measured by a Protein Assay kit (Bio-Rad Laboratories).

Electrophoretic mobility shift assay (EMSA)

The DNA-binding ability of HSF1 was analyzed by EMSA as described previously (Mosser *et al.*, 1988) with the following modifications. The *in vitro* translated products and the whole-cell extracts from RTG-2 cells were used as the protein samples. Binding reaction mixtures were incubated for 30 min on ice. Gels were run at 4°C for 3 h at 150 V, dried, and exposed on Hyperfilm-MP (Amersham Biosciences). A double-stranded synthetic HSE, which contains four inverted nGAAn repeats (5'-tcg actaGAAGcTTCTaGAAGcTTCTag-3'), was used as a probe and a competitor. The probe was end-labeled with [³²P]dCTP by the Klenow fragment of DNA polymerase I. For competition experiments, a 50-fold molar excess of unlabeled HSE oligonucleotides was added to the binding reaction mixtures.

Results

The DNA-binding ability of each HSF1 was examined by EMSA using the *in vitro* translated proteins, and gel shift bands were observed in the lanes containing epitope-tagged HSF1 (Fig. 14, lanes 5 and 8). The bands were detected at a position corresponding to the gel-shift band of heat-shocked RTG-2 cell extract (Fig. 14, lane 2), and were abolished by the addition of excess unlabeled HSE probe (Fig. 14, lanes 6 and 9). Moreover, these bands were not detected in the lanes containing epitope-tagged β -galactosidase (Fig. 14, lanes 4 and 7). This means that the gel-shift bands were not due to a factor endogenous to the *in vitro* translation mixture or to the epitope tags. Taken together, these results demonstrate that *in vitro* translated HSF1a and HSF1b, as well as endogenous HSF1 in RTG-2 cells, bind specifically to HSE consensus sequences.

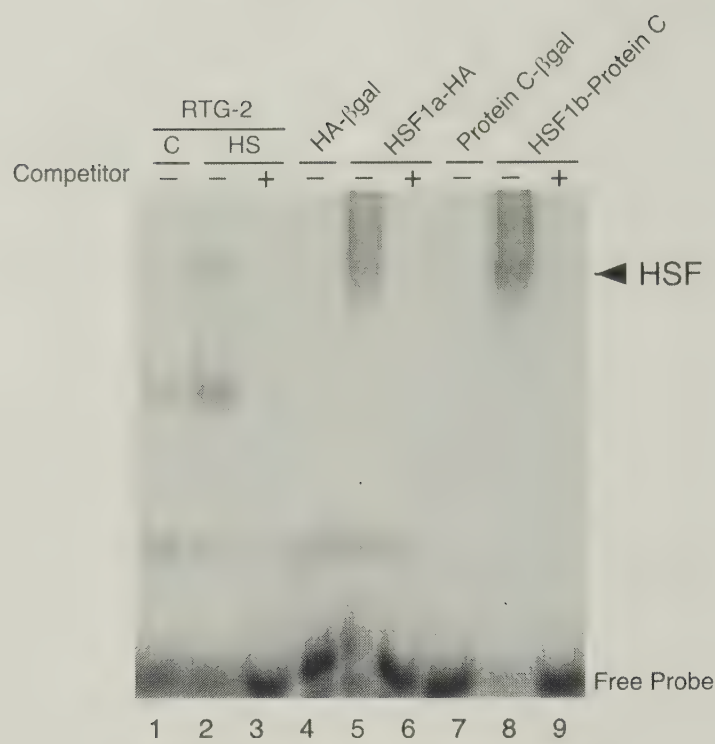


Fig. 14. EMSA of endogenous rainbow trout HSF1 and *in vitro* translated HSF1a and HSF1b. Unlabeled HSE oligonucleotides were used as a competitor and added to the binding reaction mixtures as indicated by plus signs (+). RTG-2 cells were cultured at 15°C (C) and heat shocked at 25°C for 1 h (HS). *In vitro* translated HA-β gal and Protein C-β gal were used as negative controls.

Section 5. Oligomeric state of HSF1

In the preceding section, it was demonstrated that HSF1a and HSF1b both could bind to HSE consensus sequence. It is known that mammalian and avian HSF1 binds to HSE as a homotrimer; however, rainbow trout has two HSF1 isoforms. This raises the question whether the two HSF1s form a heterotrimer or not. Thus, this section describes the analysis of oligomeric state of rainbow trout HSF1 by using the differentially epitope-tagged proteins.

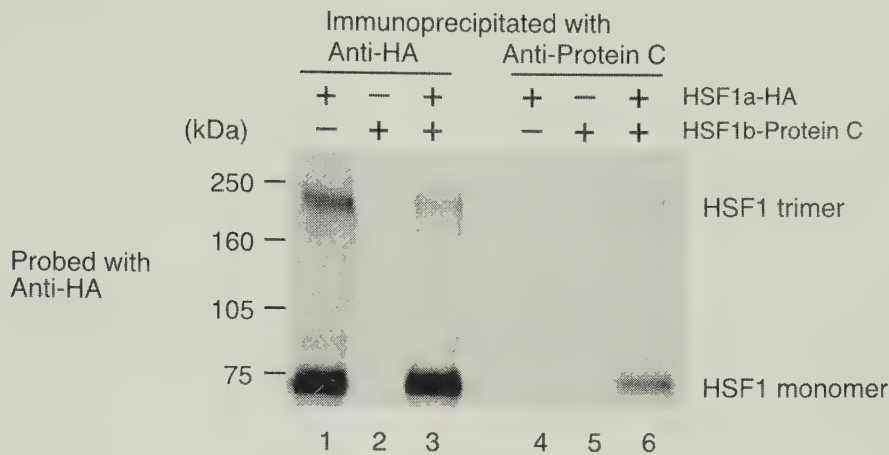
Materials and Methods

Chemical cross-linking and immunoprecipitation

In vitro translated HSF1a and HSF1b were chemically cross-linked using ethylene glycol bis

(succinimidylsuccinate) (EGS; Pierce Biotechnology, Rockford, IL, USA) as described previously (Sorger and Nelson, 1989) with the following modifications. *In vitro* translated products containing 2 mM EGS were incubated at 22°C for 30 min and then quenched by adding glycine to 50 mM at 22°C for 20 min. The samples were immunoprecipitated with anti-HA or anti-Protein C Affinity Matrix (Roche Diagnostics) according to the manufacturer's instructions. The immunoprecipitates were separated by SDS-PAGE on 6% gels. The HSF1a-HA and the HSF1b-Protein C were detected by Western blot analysis using anti-HA and anti-Protein C Ig (Roche Diagnostics), respectively, as described in Section 3 of this chapter.

A



B

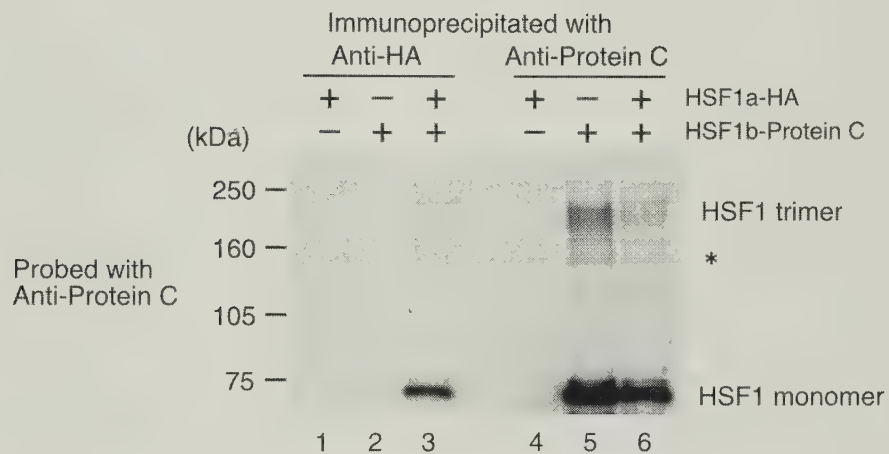


Fig. 15. Chemical cross-linking and immunoprecipitation. *In vitro* translated products containing either HSF1a or HSF1b, or both, were cross-linked using EGS and immunoprecipitated with anti-HA or anti-Protein C Ig. The immunoprecipitates were analyzed by Western blotting using antibodies against HA (A) or Protein C (B). Molecular mass markers are indicated on the left (in kDa). The asterisk indicates the band corresponding to a HSF1b dimer.

Results

To investigate whether rainbow trout HSF1s form oligomeric structures, chemical cross-linking with EGS, followed by immunoprecipitation with antibodies specific for the epitope tags, was performed. The immunoprecipitated proteins were analyzed by Western blotting. In this experiment, three *in vitro* translated products were analyzed:

HSF1a-HA, HSF1b-Protein C, and a mixture of both HSF1a-HA and HSF1b-Protein C.

Fig. 15A shows the immunoprecipitated proteins probed with anti-HA Ig. When the cross-linked proteins were immunoprecipitated with anti-HA Ig, two bands were detected in the lanes containing HSF1a-HA (Fig. 15A, lanes 1 and 3). The apparent molecular masses of the bands were ≈ 200 kDa and ≈ 70 kDa. These molecular masses correspond to the sizes of an HSF1 trimer and monomer,

respectively. This result therefore suggests that the 200- and 70-kDa products are cross-linked trimers and monomers of HSF1a-HA, respectively. Moreover, when the same cross-linked proteins were immunoprecipitated with anti-Protein C Ig, two similar bands were detected in the lane containing both HSF1a-HA and HSF1b-Protein C (Fig. 15A, lane 6). This suggests that the $\approx$ 200-kDa product is a cross-linked HSF1 trimer containing both HA and Protein C epitope tags, i.e. an HSF1 heterotrimer. Because the $\approx$ 70-kDa product is an HSF1a-HA monomer that coimmunoprecipitated with HSF1b-Protein C, this provides evidence that the two isoforms interact with each other. By contrast, no bands were observed in the lanes containing only HSF1a-HA or HSF1b-Protein C (Fig. 15A, lanes 4 and 5), verifying that the epitope tags were not interacting with themselves. These results therefore indicate that HSF1a and HSF1b interact with each other and form heterotrimers.

To confirm further the above results, the same immunoprecipitated proteins were probed with anti-Protein C Ig by using a replica membrane from the Western blotting (Fig. 15B). This analysis indicated that HSF1b also formed homotrimers and heterotrimers with HSF1a. In addition, a band corresponding to an HSF1b dimer was detected (Fig. 15B, lanes 5 and 6). Taken together, these results demonstrate that rainbow trout HSF1s form homo- and heterotrimers *in vitro*.

Section 6. Discussion

In this chapter, it was demonstrated that two distinct isoforms of HSF1 exist in rainbow trout cells. In vertebrates, *HSF1s* have been already isolated from human (Rabindran *et al.*, 1991), mouse (Sarge *et al.*, 1991), chicken (Nakai and Morimoto, 1993), *Xenopus* (Stump *et al.*, 1995), and zebrafish (R  bergh *et al.*, 2000); however, the present study is the first report of the cloning of a gene encoding HSF1 from cold-water fish species.

Using multiple sequence alignment, domain structures that are common to the HSF1 family were identified in the rainbow trout HSF1s (Fig. 8). The DNA-binding domain in both rainbow trout HSF1s is highly homologous to that of other vertebrate HSF1

(Table 6), suggesting that both HSF1a and HSF1b bind specifically to the HSE consensus sequence. As expected, both proteins did indeed bind to the HSE (Fig. 14). HSF1a and HSF1b also possess other domains conserved in the HSF1 family, i.e., HR-A/B and HR-C (Fig. 8). HR-A/B has been reported to be essential for forming HSF1 trimers through their α -helical coiled-coil structures (Clos *et al.*, 1990; Sorger and Nelson, 1989). The second hydrophobic repeat, HR-C, has been suggested to suppress trimer formation by interacting with HR-A/B under normal conditions (Rabindran *et al.*, 1993). As predicted by the presence of these domain structures, the data in this chapter demonstrate that both rainbow trout HSF1s form trimers (Fig. 15).

Furthermore, it was found that an endogenous rainbow trout HSF1 is suppressed under normal conditions but activated by heat shock in RTG-2 cells (Fig. 14, lanes 1 and 2). This stress-inducible activation of HSF1 has been observed in rainbow trout hepatocytes (Airaksinen *et al.*, 1998) and in the embryonic fibroblast cell line STE and male germ cells (Le Goff and Michel, 1999). Taken together, the results in this chapter suggest that rainbow trout HSF1s are activated to form DNA-binding trimers by heat shock in a manner similar to the activation of other vertebrate HSF1s. In addition to the conserved domain structures, both rainbow trout HSF1s contain two KRK tripeptides, which are also conserved among members of the HSF1 family (Fig. 8). The cluster of the basic residues preceding HR-A/B has been reported to be the major NLS of human HSF1 (Zuo *et al.*, 1995). Moreover, the basic peptide KRK has been reported to be a part of a bipartite type NLS in human HSF2 (Sheldon and Kingston, 1993).

In contrast to the highly conserved domains discussed above, other regions of the rainbow trout HSF1s were poorly conserved in comparison with other vertebrate HSF1s. These poorly conserved regions are illustrated in Fig. 10 as regions III and V. Regions III and V roughly correspond to domains of mammalian HSF1 that have been described by several authors (Green *et al.*, 1995; Shiet *et al.*, 1995; Zuo *et al.*, 1995), namely, the regulatory and transactivation domains, respectively. Green *et al.* (1995) have shown that the central regulatory

domain of human HSF1 regulates the function of the transactivation domain in a heat-shock inducible manner. Moreover, Newton *et al.* (1996) have suggested that the regulatory domain of human HSF1 alone is sufficient to sense heat stress. Thus, structural differences in regions III and V between rainbow trout and other vertebrate HSF1s may reflect differences in the activation temperature of HSF1. For example, human, mouse, and chicken HSF1 are activated at $\approx 42^{\circ}\text{C}$ (Morimoto, 1998), whereas rainbow trout HSF1 is activated at 25°C in RTG-2 cells (Fig. 14). Notably, regions III and V of rainbow trout HSF1s even share low similarity with the corresponding regions of zebrafish HSF1. Again, this may be related to differences in the threshold temperature for HSP induction between cold- and warm-adapted fishes, as discussed at the beginning of this chapter. Moreover, because region V of rainbow trout HSF1a shows low similarity to that of HSF1b (Table 6), transactivation may differ between the two rainbow trout HSF1s.

It was demonstrated here that each rainbow trout HSF1 is encoded by a separate gene (Fig. 11). To date, two isoforms of HSF1 generated by alternative splicing have been reported for mouse (Fiorenza *et al.*, 1995) and zebrafish (R  bergh *et al.*, 2000); however, among vertebrates, rainbow trout HSF1 is the first case of having two genetically distinct isoforms. The HSF1a and HSF1b mRNAs are coexpressed in rainbow trout tissues (Fig. 12), which suggests that both are involved in the heat shock response of rainbow trout. However, since the existence of the proteins has not been checked in the same cell, the actual protein abundance remains to be elucidated.

To characterize rainbow trout HSF1 isoforms, *in vitro* translated HSF1s having distinct epitope tags were used in this chapter. Although migration of the products was retarded in SDS-PAGE, this phenomenon may result from the poor binding of SDS to the proteins because of their acidic isoelectric point (HSF1a, 4.64; HSF1b, 4.63). As pointed out by Sarge *et al.* (1991), such retarded migration of HSF on SDS-PAGE seems to be characteristic of several HSFs that have been cloned to date. It was therefore concluded that the epitope-tagged HSF1s were successfully generated *in vitro*.

It was assumed that the *in vitro* translated HSF1s would be in the form of active trimers with DNA-binding ability because the *in vitro* translations were performed at 30°C , at which rainbow trout endogenous HSF1 is already activated *in vivo* (Airaksinen *et al.*, 1998; Le Goff and Michel, 1999). As predicted, the *in vitro* translated HSF1s did indeed possess DNA-binding ability (Fig. 14, lanes 5 and 8).

Importantly, the chemical cross-linking and immunoprecipitation experiments in this chapter showed that the two HSF1 isoforms have the ability to form heterotrimers *in vitro* (Fig. 15A, lane 6 and Fig. 15B, lane 3). Given that the two HSF1 isoforms form both homo- and heterotrimers, there are four potential assemblies of HSF1 trimer, namely, two homotrimers (a_3 and b_3) and two heterotrimers (a_2b_1 and a_1b_2). The existence of the four types of trimer may be reflected in the broad band migrating at ≈ 200 kDa in Fig. 15. On the other hand, a band corresponding to an HSF1b dimer was detected by Western blot analysis with anti-Protein C Ig (Fig. 15B, lanes 5 and 6). It remains unclear whether dimer formation is a feature only of HSF1b.

Although four HSFs, HSF1–4, have been identified in vertebrates, it has been previously stated that HSF family proteins function as homotrimers. Sarge *et al.* (1991) pointed out, however, that mouse HSF1 and HSF2 are likely to co-oligomerize because they share highly homologous oligomerization domains. Likewise, Sistonen *et al.* (1994) raised the possibility that human HSF1 and HSF2 may associate to form heterotrimers for synergistic induction of the *HSP70*. The results of this chapter concerning rainbow trout HSF1 raise the same possibility of hetero-oligomerization. If hydrophobic interactions are the major stabilizing force of HSF trimerization, it is not surprising that HSF family proteins form heteromeric complexes because they possess similar heptad repeats of hydrophobic amino acids. However, since the *in vivo* state of rainbow trout HSF1 has not been examined, it remains to be elucidated whether the HSF1 isoforms form heterotrimers *in vivo*.

Why are there two isoforms of HSF1 in rainbow trout? Although the existence of the two genes may be explained simply by ancestral tetraploidization

of salmonids, this does not rule out the possibility that the isoforms have acquired divergent functions during evolution. One possibility is that the distinct HSF1 isoforms contribute to the tissue specificity of the heat shock response. Airaksinen *et al.* (1998) reported that the induced expression of HSPs is both cell type- and tissue-specific in rainbow trout. Furthermore, it has been reported that rainbow trout HSF1, as well as mouse HSF1 (Goodson and Sarge, 1995), is activated at a lower temperature in male germ cells than in somatic cells (Le Goff and Michel, 1999). By contrast, the alternatively spliced isoforms of HSF1 are suggested to regulate the tissue-specific gene expression of HSPs in zebrafish (Råbergh *et al.*, 2000) and mouse (Sarge, 1995). In the present study, however, both HSF1a and HSF1b mRNAs were coexpressed in all rainbow trout tissues examined. Thus, the above-mentioned assemblies of HSF1 trimers, rather than transcriptional regulation of *HSF1s* themselves, may regulate the tissue specificity of the heat shock response in rainbow trout. Another possibility is that the two homotrimers and/or two heterotrimers play the role of the other HSF family members, i.e., HSF2, HSF3, and HSF4. For instance, the relationship between rainbow trout HSF1a and HSF1b may be similar to that between chicken HSF1 and HSF3. Tanabe *et al.* (1998) reported that HSF3 has a dominant role in regulating the heat shock response and directly influences HSF1 activity in chicken cells. Unfortunately, in the present study, a cDNA encoding HSF members other than HSF1 was not found. However, a cDNA encoding rainbow trout HSF2 has been reported (Le Goff *et al.*, 2004), then the relationship between HSF1 and HSF2 in this species is needed to be elucidated in future studies.

Summary and general discussion

In Chapter I, full-length cDNA clones encoding Hsp70 were isolated from rainbow trout and investigated for their mRNA expression profiles during heat stress. Consequently, two *Hsp70s*, *Hsp70a* and *Hsp70b*, were identified and found to have 98.1% identity in their deduced amino acid sequences. Southern blot analysis indicated that the two Hsp70s are encoded by distinct genes in

the genome. Northern blot analysis showed that each of *Hsp70a* and *Hsp70b* expressed two mRNA species having different sizes by heat stress in rainbow trout RTG-2 cells. The induction levels of total Hsp70b mRNAs were consistently higher than Hsp70a counterparts during heat stress, although the expression profiles of the two genes were similar to each other in temperature shift and time course experiments. Interestingly, an mRNA species with a larger molecular size was expressed only under severe heat stress not less than 28°C irrespective of *Hsp70a* and *Hsp70b*.

In Chapter II, multiple *HSPs* were isolated from RTG-2 cells and quantitatively compared for their mRNA levels between unstressed and heat-shocked cells using real-time RT-PCR analysis. Consequently, nine cDNAs encoding HSPs were isolated from heat-shocked RTG-2 cells, namely, Hsp90 β a, Hsp90 β b, Grp78, Hsp70a, Hsc70a, Hsc70b, Cct8, Hsp47, and DnaJ homolog. Quantitative RT-PCR analyses, in which *Hsp70b* isolated previously was included, showed that the mRNA accumulation levels of *Hsp70a*, *Hsp70b*, *Hsc70a*, *Hsc70b*, and *Hsp47* were significantly increased after heat shock, and the increased levels of two *Hsp70s*, *Hsp70a* and *Hsp70b*, were most conspicuous. In the case of *Hsc70s*, the increased level of *Hsc70b* was more remarkable than that of *Hsc70a*.

In Chapter III, genes encoding HSF1 were cloned from RTG-2 cells. Consequently, two distinct *HSF1s*, named *HSF1a* and *HSF1b*, were identified. The predicted amino acid sequence of *HSF1a* showed 86.4% identity to that of *HSF1b*. The two proteins contained the general structural motifs of HSF1, i.e. a DNA-binding domain, hydrophobic heptad repeats and nuclear localization signals. Southern blot analysis showed that each HSF1 is encoded by a distinct gene. The two HSF1 mRNAs were coexpressed in unstressed RTG-2 cells and in various tissues. In EMSA, each *in vitro* translated HSF1 bound to the HSE. Chemical cross-linking and immunoprecipitation analysis showed that HSF1a and HSF1b form heterotrimers as well as homotrimers. Taken together, these results demonstrate that in rainbow trout cells there are two distinct HSF1 isoforms that can form heterotrimers, suggesting that a unique molecular

mechanism underlies the stress response in tetraploid and/or cold-water fish species.

The present study demonstrated that, in rainbow trout, at least some *HSP* and *HSF* have duplicate genes attributed to ancestral tetraploidization. The genes found to be duplicated in the present study are summarized as follows: *Hsp90 β* , *Hsp70*, *Hsc70* and *HSF1*. Since the other *HSP* may have possible paralogs as discussed in Section 3 of Chapter II, it is apparent that the comprehensive identification of duplicate genes is prerequisite to accurately examining gene expression profiles of rainbow trout cells. Meanwhile, HSPs are induced in fish cells by a variety of stresses, including industrial effluents, polycyclic aromatic hydrocarbons, several metals such as copper, zinc and mercury, pesticides, and arsenite as well as heat (Iwama *et al.*, 2004). Thus, although the present study focused on heat stress, it is likely that the mRNA expression profiles of HSPs differ among stressors. It is interesting to compare the differences of the profiles for utilizing *HSPs* as a biomarker of various environmental stresses.

Further, the present study demonstrated that two distinct isoforms of HSF1 can form heterotrimers as well as homotrimers *in vitro*. This finding suggests that a unique molecular mechanism, which functions through two distinct HSF1 isoforms, underlies the stress response in rainbow trout. However, the *in vivo* functional difference between HSF1a and HSF1b remains to be elucidated. Since the lower activation temperature of rainbow trout HSF1 is a unique feature among vertebrate counterparts, a detailed comparison of rainbow trout and other vertebrate HSF1s will lead to further insight into the activation mechanism of the transcription factor.

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References

- Airaksinen S., Råbergh C. M. I., Sistonen L. and Nikinmaa M., 1998: Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J. Exp. Biol.*, **201**, 2543-2551.
- Basu N., Todgham A. E., Ackerman P. A., Bibeau M. R., Nakano K., Schulte P. M. and Iwama G. K., 2002: Heat shock protein genes and their functional significance in fish. *Gene*, **295**, 173-183.
- Clos J., Westwood J. T., Becker P. B., Wilson S., Lambert K. and Wu C., 1990: Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. *Cell*, **63**, 1085-1097.
- Currie S., Tufts B. L. and Moyes C. D., 1999: Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *Am. J. Physiol.*, **276**, R990-R996.
- Dellavalle R. P., Petersen R. and Lindquist S., 1994: Preferential deadenylation of Hsp70 mRNA plays a key role in regulating Hsp70 expression in *Drosophila melanogaster*. *Mol. Cell. Biol.*, **14**, 3646-3659.
- Fiorenza M. T., Farkas T., Dissing M., Kolding D. and Zimarino V., 1995: Complex expression of

- murine heat shock transcription factors. *Nucleic Acids Res.*, **23**, 467-474.
- Gething M.-J. (ed.), 1997a: Guidebook to Molecular Chaperones and Protein-Folding Catalysts, Oxford University Press, Oxford.
- Gething M.-J., 1997b: Mammalian BiP, in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 59-64.
- Goodson M. L. and Sarge K. D., 1995: Regulated expression of heat shock factor 1 isoforms with distinct leucine zipper arrays via tissue-dependent alternative splicing. *Biochem. Biophys. Res. Commun.*, **211**, 943-949.
- Green M., Schuetz T. J., Sullivan E. K. and Kingston R. E., 1995: A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. *Mol. Cell. Biol.*, **15**, 3354-3362.
- Hawkins A. J. S., 1996: Temperature adaptation and genetic polymorphism in aquatic animals, in "Animals and Temperature: Phenotypic and Evolutionary Adaptation" (ed. by Johnston I. A. and Bennett A. F.), Cambridge University Press, Cambridge, pp. 103-125.
- Hightower L. E. and Leung S.-M., 1997: Mammalian Hsc70 and Hsp70 proteins, in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 53-58.
- Hightower L. E. and Renfro J. L., 1988: Recent applications of fish cell culture to biomedical research. *J. Exp. Zool.*, **248**, 290-302.
- Hirayoshi K., Kudo H., Takechi H., Nakai A., Iwamatsu A., Yamada K. M. and Nagata K., 1991: HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. *Mol. Cell. Biol.*, **11**, 4036-4044.
- Iwama G. K., Afonso L. O. B., Todgham A., Ackerman P. and Nakano K., 2004: Are hsp's suitable for indicating stressed states in fish? *J. Exp. Biol.*, **207**, 15-19.
- Kaya C. M., 1978: Thermal resistance of rainbow trout from a permanently heated stream and two hatchery strains. *Prog. Fish-Cult.*, **40**, 138-142.
- Kothary R. K. and Candido E. P. M., 1982: Induction of a novel set of polypeptides by heat shock or sodium arsenite in cultured cells of rainbow trout, *Salmo gairdnerii*. *Can. J. Biochem.*, **60**, 347-355.
- Kothary R. K., Burgess E. A. and Candido E. P. M., 1984a: The heat-shock phenomenon in cultured cells of rainbow trout hsp70 mRNA synthesis and turnover. *Biochim. Biophys. Acta*, **783**, 137-143.
- Kothary R. K., Jones D. and Candido E. P. M., 1984b: 70-Kilodalton heat shock polypeptides from rainbow trout: characterization of cDNA sequences. *Mol. Cell. Biol.*, **4**, 1785-1791.
- Kubota H., Matsumoto S., Yokota S., Yanagi H. and Yura T., 1999: Transcriptional activation of mouse cytosolic chaperonin CCT subunit genes by heat shock factors HSF1 and HSF2. *FEBS Lett.*, **461**, 125-129.
- Kubota H. and Willison K., 1997: CCT θ , in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 225-226.
- Le Goff P., Le Drian Y., Le Peron C., Le Jossic-Corcus C., Ainouche A. and Michel D., 2004: Intracellular trafficking of heat shock factor 2. *Exp. Cell Res.*, **294**, 480-493.
- Le Goff P. and Michel D., 1999: HSF1 activation occurs at different temperatures in somatic and male germ cells in the poikilotherm rainbow trout. *Biochem. Biophys. Res. Commun.*, **259**, 15-20.
- Lindquist S. and Craig E. A., 1988: The heat-shock proteins. *Annu. Rev. Genet.*, **22**, 631-677.
- Lund S. G., Phillips M. C., Moyes C. D. and Tufts B. L., 2000: The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.*, **203**, 2219-2228.
- Miao B., Davis J. and Craig E. A., 1997: The Hsp70 family—an overview, in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 3-13.
- Morimoto R. I., 1998: Regulation of the heat shock transcriptional response: cross talk between

- a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, **12**, 3788-3796.
- Mosser D. D., Heikkilä J. J. and Bols N. C., 1986: Temperature ranges over which rainbow trout fibroblasts survive and synthesize heat-shock proteins. *J. Cell. Physiol.*, **128**, 432-440.
- Mosser D. D., Theodorakis N. G. and Morimoto R. I., 1988: Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. *Mol. Cell. Biol.*, **8**, 4736-4744.
- Mosser D. D., Van Oostrom J. and Bols N. C., 1987: Induction and decay of thermotolerance in rainbow trout fibroblasts. *J. Cell. Physiol.*, **132**, 155-160.
- Munro S. and Pelham H. R. B., 1987: A C-terminal signal prevents secretion of luminal ER proteins. *Cell*, **48**, 899-907.
- Nagata K., 1997: Vertebrate Hsp47, in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 465-467.
- Nakai A. and Morimoto R. I., 1993: Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol. Cell. Biol.*, **13**, 1983-1997.
- Newton E. M., Knauf U., Green M. and Kingston R. E., 1996: The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. *Mol. Cell. Biol.*, **16**, 839-846.
- Ohno S., Wolf U. and Atkin N. B., 1968: Evolution from fish to mammals by gene duplication. *Hereditas*, **59**, 169-187.
- Page R. D., 1996: TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.*, **12**, 357-358.
- Palmisano A. N., Winton J. R. and Dickhoff W. W., 1999: Sequence features and phylogenetic analysis of the stress protein hsp90 α in chinook salmon (*Oncorhynchus tshawytscha*), a poikilothermic vertebrate. *Biochem. Biophys. Res. Commun.*, **258**, 784-791.
- Palti Y., Gahr S. A., Hansen J. D. and Rexroad C. E., III, 2004: Characterization of a new BAC library for rainbow trout: evidence for multi-locus duplication. *Anim. Genet.*, **35**, 130-133.
- Pearson D. S., Kulyk W. M., Kelly G. M. and Krone P. H., 1996: Cloning and characterization of a cDNA encoding the collagen-binding stress protein hsp47 in zebrafish. *DNA Cell Biol.*, **15**, 263-272.
- Pirkkala L., Nykänen P. and Sistonen L., 2001: Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J.*, **15**, 1118-1131.
- Rabindran S. K., Giorgi G., Clos J. and Wu C., 1991: Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci. USA*, **88**, 6906-6910.
- Rabindran S. K., Haroun R. I., Clos J., Wisniewski J. and Wu C., 1993: Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science*, **259**, 230-234.
- Rise M. L., Von Schalburg K. R., Brown G. D., Mawer M. A., Devlin R. H., Kuipers N., Busby M., Beetz-Sargent M., Alberto R., Gibbs A. R., Hunt P., Shukin R., Zeznik J. A., Nelson C., Jones S. R. M., Smailus D. E., Jones S. J. M., Schein J. E., Marra M. A., Butterfield Y. S. N., Stott J. M., Ng S. H. S., Davidson W. S. and Koop B. F., 2004: Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. *Genome Res.*, **14**, 478-490.
- Ryan J. A. and Hightower L. E., 1996: Stress proteins as molecular biomarkers for environmental toxicology. *EXS*, **77**, 411-424.
- Råbergh C. M. I., Airaksinen S., Soitamo A., Björklund H. V., Johansson T., Nikinmaa M. and Sistonen L., 2000: Tissue-specific expression of zebrafish (*Danio rerio*) heat shock factor 1 mRNAs in response to heat stress. *J. Exp. Biol.*, **203**, 1817-1824.
- Saitou N. and Nei M., 1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406-425.
- Samples B. L., Pool G. L. and Lumb R. H., 1999: Polyunsaturated fatty acids enhance the heat induced stress response in rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Comp. Biochem. Physiol.*, **123B**, 389-397.
- Sarge K. D., 1995: Male germ cell-specific alteration in temperature set point of the cellular stress

- response. *J. Biol. Chem.*, **270**, 18745–18748.
- Sarge K. D., Zimarino V., Holm K., Wu C. and Morimoto R. I., 1991: Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability. *Genes Dev.*, **5**, 1902–1911.
- Sathiyaa R., Campbell T. and Vijayan M. M., 2001: Cortisol modulates HSP90 mRNA expression in primary cultures of trout hepatocytes. *Comp. Biochem. Physiol.*, **129B**, 679–685.
- Satoh M., Hirayoshi K., Yokota S., Hosokawa N. and Nagata K., 1996: Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. *J. Cell Biol.*, **133**, 469–483.
- Scheibel T. and Buchner J., 1997: The HSP90 family—an overview, in “Guidebook to Molecular Chaperones and Protein-Folding Catalysts” (ed. by Gething M.-J.), Oxford University Press, Oxford, pp. 147–151.
- Scheufler C., Brinker A., Bourenkov G., Pegoraro S., Moroder L., Bartunik H., Hartl F. U. and Moarefi I., 2000: Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell*, **101**, 199–210.
- Sheldon L. A. and Kingston R. E., 1993: Hydrophobic coiled-coil domains regulate the subcellular localization of human heat shock factor 2. *Genes Dev.*, **7**, 1549–1558.
- Shi Y., Kroeger P. E. and Morimoto R. I., 1995: The carboxyl-terminal transactivation domain of heat shock factor 1 is negatively regulated and stress responsive. *Mol. Cell. Biol.*, **15**, 4309–4318.
- Sistonen L., Sarge K. D. and Morimoto R. I., 1994: Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. *Mol. Cell. Biol.*, **14**, 2087–2099.
- Somorjai I. M. L., Danzmann R. G. and Ferguson M. M., 2003: Distribution of temperature tolerance quantitative trait loci in Arctic charr (*Salvelinus alpinus*) and inferred homologies in rainbow trout (*Oncorhynchus mykiss*). *Genetics*, **165**, 1443–1456.
- Sonna L. A., Fujita J., Gaffin S. L. and Lilly C. M., 2002: Effects of heat and cold stress on mammalian gene expression. *J. Appl. Physiol.*, **92**, 1725–1742.
- Sorger P. K. and Nelson H. C., 1989: Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell*, **59**, 807–813.
- Sreedhar A. S., Kalmár É., Csermely P. and Shen Y.-F., 2004: Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett.*, **562**, 11–15.
- Stump D. G., Landsberger N. and Wolffe A. P., 1995: The cDNA encoding *Xenopus laevis* heat-shock factor 1 (XHSF1): nucleotide and deduced amino-acid sequences, and properties of the encoded protein. *Gene*, **160**, 207–211.
- Takechi H., Hosokawa N., Hirayoshi K. and Nagata K., 1994: Alternative 5' splice site selection induced by heat shock. *Mol. Cell. Biol.*, **14**, 567–575.
- Tanabe M., Kawazoe Y., Takeda S., Morimoto R. I., Nagata K. and Nakai A., 1998: Disruption of the HSF3 gene results in the severe reduction of heat shock gene expression and loss of thermotolerance. *EMBO J.*, **17**, 1750–1758.
- Thompson J. D., Higgins D. G. and Gibson T. J., 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
- Thorgaard G. H., Bailey G. S., Williams D., Buhler D. R., Kaattari S. L., Ristow S. S., Hansen J. D., Winton J. R., Bartholomew J. L., Nagler J. J., Walsh P. J., Vijayan M. M., Devlin R. H., Hardy R. W., Overturf K. E., Young W. P., Robison B. D., Rexroad C. and Palti Y., 2002: Status and opportunities for genomics research with rainbow trout. *Comp. Biochem. Physiol.*, **133B**, 609–646.
- Wolf K. and Quimby M. C., 1962: Established eurythermic line of fish cells *in vitro*. *Science*, **135**, 1065–1066.
- Wu C., 1995: Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.*, **11**, 441–469.
- Yamashita M., Hirayoshi K. and Nagata K., 2004: Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates. *Gene*, **336**, 207–218.

- Yokoyama Y., Hashimoto H., Kubota S., Kinoshita M., Toyohara H., Sakaguchi M., Tanaka M., Seikai T. and Kanamori M., 1998: cDNA cloning of heat-inducible HSP70, a 70.6 kDa heat shock protein, in Japanese flounder *Paralichthys olivaceus*. *Fish. Sci.*, **64**, 964-968.
- Zafarullah M., Wisniewski J., Shworak N. W., Schieman S., Misra S. and Gedamu L., 1992: Molecular cloning and characterization of a constitutively expressed heat-shock-cognate hsc71 gene from rainbow trout. *Eur. J. Biochem.*, **204**, 893-900.
- Zuo J., Rungger D. and Voellmy R., 1995: Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.*, **15**, 4319-4330.

和文要旨

ブリ (*Seriola quinqueradiata*) の産卵, 回遊生態及びその研究課題・手法について

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ブリ *Seriola quinqueradiata* は日本各地で定置網, 巻き網などの重要な漁獲対象になっており, 漁況予報の精度向上, 適正な資源管理が求められている。本稿ではブリの産卵生態, 回遊生態についてレビューを行い, 併せて上記の要望に応えるためにはどのような研究をどのような手法で遂行するのが適当であるか検討を行った。ブリの産卵生態は, 卵仔稚魚調査を中心とした既往知見の整理の他に, 仔稚魚の成長, 親魚の成熟状況から見た産卵生態についてまとめを行った。回遊については成長段階によって様式が異なり成魚では大規模な南北回遊がみられること, 越冬域をはじめとする分布域は海洋環境に依存していると考えられた。漁況予報の精度向上のためには回遊と環境の関係を解明することが必要と考えられ, その研究を遂行するための手法についても検討を行った。

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新規防汚物質の水域環境における挙動, 汚染状況及び水生生物に対する有害性並びに水域生態系に対する予備的リスク評価

山田 久 (中央水産研究所)

有機スズ化合物 (TBT) の代替新規防汚物質の水域環境における挙動や濃度, 水生生物に対する有害性を既往文献情報に基づき取りまとめるとともに, 得られた情報により水域生態系に対するリスクを予察した。新規防汚物質の有害性は TBT に匹敵するが, 分解しやすく, 環境リスクは TBT より小さいと推察された。しかし, Irgarol 1051及び Sea Nine 211の海水中濃度が無影響濃度を超えるため, リスク評価研究の深化の必要性を指摘した。

No.21, 31-45 (2007)

ニジマス熱ショックタンパク質遺伝子の発現調節機構に関する研究

尾島信彦 (中央水産研究所)

本研究はニジマス HSP の構造と遺伝子発現特性, ならびに発現調節機構の解明を目的とした。結果, 重複した *Hsp70* の存在と, 熱ストレスの強さによる各 mRNA の発現パターン変化を明らかにした。*Hsp70*, *Hsc70*, *Hsp47* の mRNA 蓄積量は熱ショックにより有意に増加した。また HSF1アイソフォーム遺伝子を 2 種類単離し, 両タンパク質とも HSP ファミリー遺伝子の熱誘導性転写調節への関与が示唆された。

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